The role of abscisic acid in fruit development in tomato

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"We have thus before us the interesting case of plant tissues, the tissues of the future fruit, which are capable of growing but which suddenly stop doing so at the time the flower opens. This fact occurs so usually that the attention of most plant physiologists has been devoted to what factors actually cause fruits to develop after flowering, taking for granted that ovary growth necessarily has been stopped at that time. It is the authors opinion, however, that the important question is not so much "Why does the ovary grow into a fruit?" but rather "Why doesn't it always grow into a fruit?"

Nitsch J.P. (1952) Plant hormones in the development of fruits, Quarterly reviews of Biology 27: 33-57.

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Chapter 1

General Introduction

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General introduction

Species sustainability and food

The topic of this thesis, fruit development, has been studied in tomato for a very long time. Several genetic and molecular tools have been developed in the past years, making tomato a good model species to study fruit development. Moreover, tomato is a member of the Solanaceae plant family, which also comprizes important other crop species such as potato, pepper and egg-plant. There are several reasons why fruit development is and has been studied. Importantly, it can provide us with better insight in two main topics: species sustainability and food.

Fruits are often eaten by animals. This is not remarkable, since some plants produce fruits with the purpose of being eaten. Via this way they can disperse their seeds. Additionally, the fruit is protecting the seeds during their development. Fruit development is thus tightly linked with seed formation, seed dispersal and consequently species sustainability.

"The World Food Problem" has been a familiar phrase from the seventies, but it is still a matter of great concern in our present times. Problems of equal distribution of food, sustainable land use, but also of increased food production to equalize the increase in world population, still are unresolved issues. Plant growth and crop yield are at the bases of an increased food production. Most of the world nutritional plant products are fruits and seeds, such as tomato, pepper, maize and rice. Knowledge on fruit development might thus be of importance to answer to problems of food supplies now and in the future.

Compelling evidence indicates that during fruit development all major hormone classes in plants interact with each other. Therefore, fruit development is also interesting, because hormonal cross talk can be studied in this system. During the first step of fruit development, fruit initiation, at least three hormones, auxin, gibberellin and cytokinin, play a role in the initiation of cell division after successful fertilization. The signal components and the mechanisms by which these hormones work together during early fruit development are however largely unknown and thus they remain an interesting object to study.

Fruits

Fruits are mostly formed as an out-growth of the female reproductive organ; the mature ovary (Fig. 1). Sometimes however, other flower structures, such as the receptacle in strawberry or the coalesced sepal, petal and stamen base, as in apple and for example banana, also contribute to the final fruit (Crane, 1964). Fruits can be divided into dry fruits and fleshy fruits. Dry fruit at maturity can either open (dehiscent dry fruit), or stay closed (indehiscent dry fruit). Arabidopsis is probably the most famous example of a species with dehiscent dry fruits. In fleshy fruits the fruit wall has become soft and it has developed more extensively. Tomato is an example of a species with fleshy fruits (Crane, 1964). Both dry and fleshy fruit are consumed, examples are pea and tomato, but in the case of a dry fruit it is mainly the seeds that are nutritional.

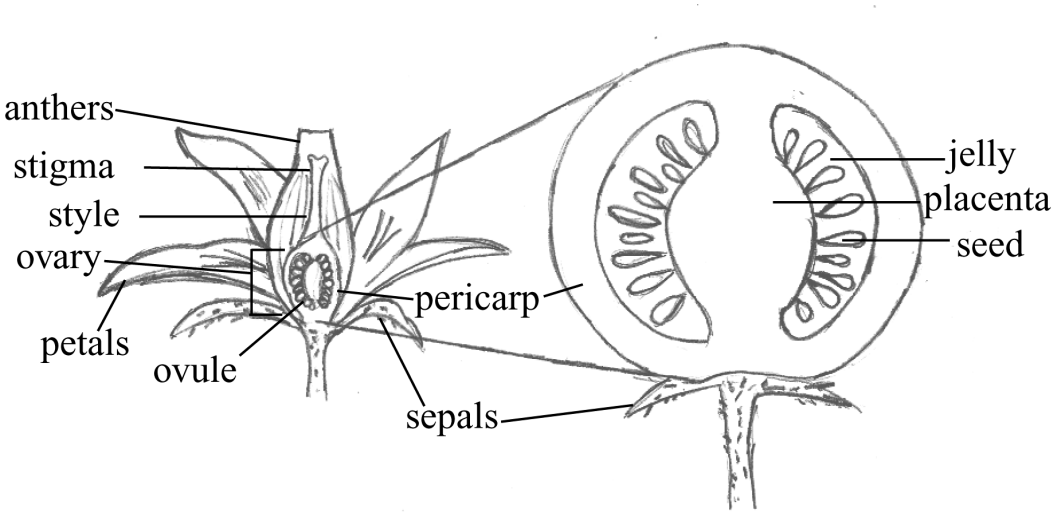

Fig. 1: Schematic representation of the different parts of the tomato flower and fleshy tomato fruit. During fruit development the ovary wall will grow into the fruit wall, both called pericarp, and the ovules will form the seeds.

Figure 1 shows a schematic representation of a tomato flower and tomato fruit. The different parts of the ovary and their equivalent parts in the fruit are depicted. It is visible that the ovary wall (pericarp) will make up the major part of the fruit, the fruit wall (also called pericarp) while the fertilized ovules will form the seeds. The central part of the ovary, the placenta, will grow around the seeds and near the end of fruit development the jelly will be produced. The process of fruit development starts with flower and ovary development. The importance of ovary development is sometimes neglected when fruit development is

discussed. A classification of the following phases of fruit development was made among others by Gillaspy *et al.* (1993), the different phases are schematically depicted in figure 2. The first phase described after ovary development is called fruit set or fruit initiation. It is defined as: the stage at which the ovary is committed to abort or to proceed with further cell division and fruit development. The next phase is characterized by a very strong increase in cell number and is therefore called cell division phase. In tomato this phase normally takes between seven and ten days (Bohner and Bangerth 1988; Mapelli *et al.* 1978). After cell division the growth of the fruit is mainly caused by cell enlargement. This phase is also referred to as the rapid growth phase and it continues for six to seven weeks in tomato. The final phase is the ripening phase during which tissue softening, carotenoid accumulation and sugar and acid accumulation take place, in order to provide the fruit with an appealing appearance and taste (Gillaspy *et al.* 1993).

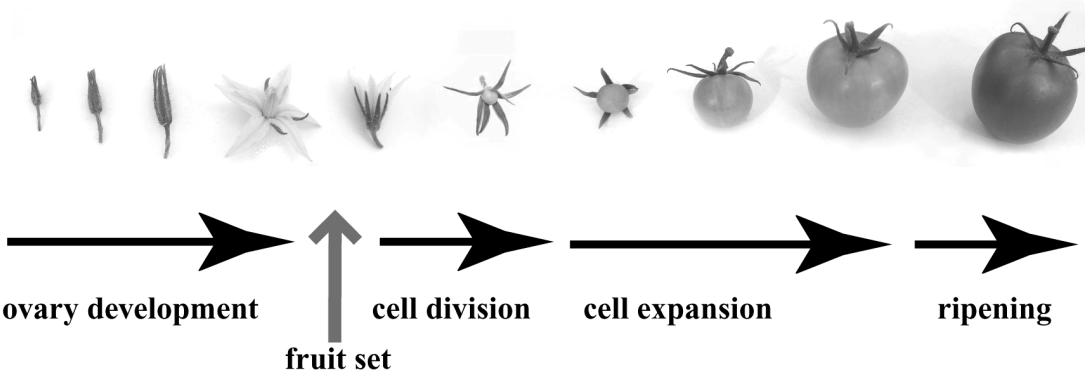

Fig. 2: Tomato fruit development can be divided in five phases: ovary development, fruit set, cell division phase, cell expansion phase and ripening.

The beginning of the fruit: the mature ovary

As mentioned before flower development might be seen as the first phase of fruit development. Indeed, proper flower development is indispensable for fruit development. The flower is formed by four whorls of organs. There are two rings of sterile organs (sepals and petals). The sepals protect the flower bud during its development, and the brightly coloured petals (corolla) are often used to attract pollinators. More inward two rings of fertile organs, anthers and carpel, are formed. The latter is comprized of ovary, style and stigma. The reproductive organs of the flower produce the gametes that need to fuse during the fertilization process. If development of the gametes or of other parts of the flower that facilitate the fertilization process is disturbed, fertilization will be less efficient or absent, leading to reduced fruit formation.

Many cell divisions in the ovary are made before it reaches its mature state at flower anthesis. It is known that factors produced by the tissue surrounding the developing ovary are required for triggering and maintaining these cell divisions (Gillaspy *et al*. 1993). Strikingly, when the ovary is mature there is a temporary reduction in cell division activity. Genes involved in cell division, such as cyclin dependent kinases (CDK's), cyclins (Cyc's), or *PROLIFERATING CELL NUCLEAR ANTIGEN* (*PCNA*) are relatively lowly expressed in the mature ovary (Kvarnheden *et al*. 2000; Vriezen *et al*. 2008). Additionally, it was concluded from a kinematic analysis of ovary growth in *Lilium longiflorum* that at maturity the ovary ceases growth (Crone and Lord 1991). Still, little attention has been given to this remarkable feature, which has already been described well more than 50 years ago among others by J.P. Nitsch (1952), *see first page*

Growth and development in the mature ovary thus cease upon flower maturity awaiting a positive signal to continue growth. One might compare this temporary quiescent state of the ovary with dormancy as it is found in seeds, axillary or apical buds. The definition of dormancy is highly debated, and the wider or stricter it is used, the more or less processes will fit the definition. A fairly wide definition is that dormancy is a period of arrested plant growth often closely associated with environmental conditions. Obviously, dormancy is associated with reduced levels of cell division and control of dormancy must at some level have an effect on cell cycle regulation (Olsen 2003). In the mature ovary reduced cell division activity is observed. Moreover, homologues of genes that proved to be excellent markers for dormancy (Stafstrom *et al.* 1998) are highly expressed in mature ovaries (*DRM1* and *DRM3*, Vriezen *et al*. 2008). Taken together these observations suggest that the mature ovary before pollination is in a dormancy-like state, which is one of the hypotheses that I approached in my thesis.

Fruit set

Less than two days after pollination transcript and protein levels of cell cycle genes are activated again (Joubes *et al.* 1999; Kvarnheden *et al*. 2000; Vriezen *et al*. 2008). Additionally, the mitotic index increases three fold from anthesis to two days after anthesis (Joubes *et al.* 1999). Pollination and fertilization produce most likely the first positive signals, which induce cell divisions and development in the ovary again after its dormancy-like state. The discussion whether pollination and pollen tube growth or fertilization induce fruit initiation has been vivid for a very long time.

In 1902 Massart placed dead pollen upon the stigma of an orchid and observed slight growth of the ovary. His experiments were followed by Fitting in 1909 with similar results (Gustafson 1937; Nitsch 1952). Later experiments with dead pollen were sometimes effective in inducing fruit set. This seems to suggest that pollen tube growth and fertilization are not required for fruit initiation. Around the same time, extracts of pollen were used to induce fruit set, and in several cases initiation of ovary growth was observed (Gustafson 1937, 1938; Nitsch 1952). It was concluded that in the pollen extracts, one or more substances were present that could induce fruit set (Gustafson 1937). Nowadays, it is accepted that stimulating growth substances, gibberellin and auxin, are present in the pollen and produced during pollen tube growth (Srivastava and Handa 2005). However, in early experiments, further fruit development was not observed after the first fruit initiation induced by pollen extracts. Thus, it seemed as if embryo or seed formation produced some additional substances to maintain growth (Gustafson 1937). Additionally, it is known that the size of fruits is proportional to number of developing seeds (Nitsch 1952; Gillaspy *et al.* 1993), indicating that seed development stimulates fruit growth. Altogether, it is most likely that pollen derived growth stimulators, together with growth stimulators produced during pollen tube growth, and fertilization induced signals, stimulate fruit initiation (Gillaspy, *et al*. 1993), while growth stimulators produced during embryo and seed formation, are important for further fruit growth.

Hormones in fruit set

The involvement of growth regulators (hormones) during fruit set has been discovered a long time ago. In the late thirties, it was observed that application of auxins led to fruit initiation in several species (Gustafson 1936). Somewhat later, also gibberellins, which were discovered in plants only in 1957 (Phinney *et al.* 1957), were identified as positive growth stimulators in fruit set. In tomato it was found that gibberellins could even induce fruit set at lower concentrations than auxin could (Wittwer *et al*. 1957), although fruits size was generally smaller than the fruit size of auxin-induced fruits (Crane 1964). It was therefore suggested that both auxin and gibberellin are part of the signaling cascade leading to fruit set. Accordingly, the endogenous levels of auxins and gibberellins were found to increase in tomato ovaries from anthesis onwards (Koshioka *et al*. 1994: Mapelli *et al.* 1978).

Gibberellin levels are peaking around six days after pollination and decrease thereafter only to give a smaller peak twenty-five days after pollination (Koshioka *et al*. 1994; Mapelli et *al.* 1978). Additionally, it was found that *LeCPS* and *Le20ox-1, -3* (GA-biosynthesis genes)

were expressed at higher levels in small tomato fruits as compared to unpollinated ovaries (Rebers *et al*. 1999; Serrani *et al*. 2007). Moreover, changed expression of key regulators of the gibberellin signaling pathway often results in parthenocarpic fruits. These fruits are produced without fertilization and thus without seed formation. It was for instance shown that the depletion of the *SlDELLA*, a negative regulator of the gibberellin response in tomato, was sufficient to overcome the growth arrest normally imposed on the ovary at anthesis (Marti *et al*. 2007). Altogether this supports that gibberellin is one of the positive signals leading to fruit set in tomato.

Auxin levels also increased directly after pollination, concomitant with the increase in gibberellin levels (Mapelli *et al.* 1978). Increasing auxin levels by over-expression of the auxin biosynthesis gene, *iaaM*, resulted in parthenocarpic fruit set in tomato and other species (Mezzetti *et al*. 2004; Rotino *et al.* 1997, 2005). Additionally, also key elements of the auxin-signaling pathway were shown to be important for fruit set. *AUXIN RESPONSE FACTOR 8* loss of function mutants caused parthenocarpic fruit set in Arabidopsis, while *AUXIN RESPONSE FACTOR 7* RNAi-silenced lines in tomato also had parthenocarpic fruits (Goetz *et al*. 2007; De Jong *et al*. 2008). Moreover, also the interaction partners of ARFs (Aux/IAA-proteins) were shown to be important players in fruit set. Down-regulation of *IAA9* also resulted in parthenocarpic fruit set in tomato (Wang *et al*. 2005). Thus, besides GA, also auxin has been implicated in the induction of tomato fruits.

The sequential order of the two signals is not fully understood. It has been measured that the auxin level increases after gibberellin treatment, as it does after pollination (Sastry and Muir, 1963), which suggests that an increase in gibberellin levels is the first signal. However, substantially more evidence has shown that auxin can stimulate GA biosynthesis and alter GA signal transduction (Frigerio *et al*. 2006; Fu and Harberd 2003; Ozga *et al.* 2003; Ross and o'Neill 2001; Vanhuizen *et al.* 1995), indicating the opposite. Thus, there seems to be considerable interaction between gibberellins and auxin during fruit set. However, genetic studies in Arabidopsis also revealed that there are independent pathways for auxin and GA to induce fruit set (Vivian-smith and Koltunow 1999). Sequential but also parallel action of gibberellins and auxin seem to be part of a signal transduction chain that leads to fruit set.

In our lab research undertaken to discover some of the molecular players during fruit set in tomato resulted in the discovery of many genes related to auxin and gibberellin signaling (Vriezen *et al*. 2008). However, surprisingly, also many genes related to stress and abscisic acid (ABA) were discovered. These ABA response- and signaling-genes were highly expressed in the mature ovary before pollination and reduced significantly after pollination,

when fruit development was initiated (Vriezen *et al*. 2008). The research presented in this thesis was aimed to gain more insight in the role of abscisic acid during this process.

Abscisic acid

Of the five classical hormones, auxin, gibberellin, cytokinin, ethylene and ABA, the latter was the last to be discovered in 1963. It is produced by cleavage of carotenoids via a pathway which has been largely dissected (Fig. 3). The first committed step of ABA biosynthesis is the production of xanthoxin from 9-*cis*-violaxanthin or 9-*cis*-neoxanthin by enzymes called 9-*cis*-epoxycarotenoid-dioxygenases (NCEDs). Xanthoxin is transported from the plastids into the cytoplasm where it is further transformed into abscisic aldehyde. Aldehyde oxidases then catalyze the final step of ABA biosynthesis. There also seems to be a shunt pathway via abscisic alcohol (Cutler and Krochko 1999; Schwarz *et al.* 2003). ABA is mainly catabolized by ABA-8`-hydroxylases which produce 8`-OH-ABA, phaseic acid (PA) and dihydrophaseic acid (DPA) (Nambara and Marion-Poll 2005, Fig. 3). In addition to biosynthesis and catabolism, ABA can circulate throughout the plant or stored as an inactive glucose ester conjugate (G-ABA) and then released into an active form by apoplastic or endoplasmicreticulum localized β-glucosidases (Wasilewska *et al.* 2008). ABA perception sites are known to be localized both on the inside and outside of the cell (Finkelstein *et al.* 2002; Wasilewska *et al.* 2008), but remain largely undiscovered. Few intra-cellular and extra-cellular ABA- receptors were claimed (Liu *et al.* 2007a; Razem *et al.* 2006; Shen *et al.* 2006), but inaccuracies with the ABA binding assay have lead to the withdrawal of the paper describing one of these receptors, FCA, and raised doubts about the others (Risk *et al.* 2008). Although, the newly identified GTG1 and GTG2 proteins seem to be genuine membrane localized ABA-receptors (Pandey *et al.* 2009), it is possible that other ABA receptors will be identified in the future. Besides multiple perception sites for ABA there also seems to be substantial evidence for the existence of multiple redundant ABA signaling cascades (Finkelstein *et al.* 2002; Nambara and Marion-Poll 2003). Moreover, ABA interacts with many other hormones but also with sugar- and light signaling, indicating that ABA signaling resembles more of a signaling web than a linear pathway (Finkelstein *et al.* 2002; Nambara and Marion-Poll 2003). Many regulatory factors of ABA signaling, including transcription factors and second messengers, have been identified but a slightly fragmented view of ABA signaling remains.

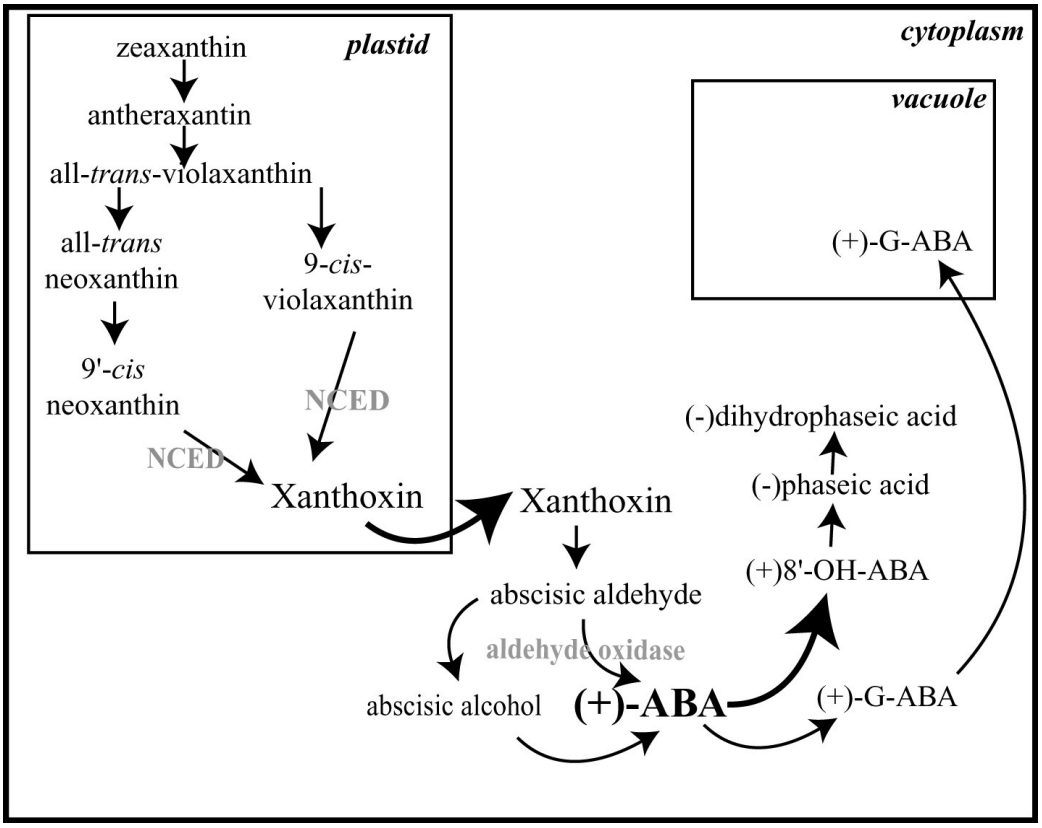

Fig. 3: Schematic overview of ABA biosynthesis and catabolism, adapted from Cutler and Krochko (1999). ABA is produced from carotenoids via cleavage in the plastids. The first committed step (formation of xanthoxin from 9-*cis*-violaxanthin or 9-*cis*-neoxanthin) is performed by 9-*cis*-epoxycarotenoid dioxygenases (NCEDs). Xanthoxin is transported into the cytoplasm and further transformed into ABA, the final step is performed by aldehyde oxidases. ABA is catabolized mainly by hydroxylation, via 8`-OH-ABA it is transformed into phaseic acid and diphaseic acid. ABA can also be transported or stored as glucose-ester- conjugate (G-ABA).

The two most pronounced functions of ABA are related to abiotic stress and seed dormancy. ABA is renowned for its function during water stress resulting from e.g. drought or salt in the environment (Seki *et al*. 2007; Wasilewska *et al.* 2008). ABA can induce stomatal closure and can thereby reduce water loss, but it also induces a battery of genes that presumably serve to protect the cells from the resulting oxidative damage (Seki *et al*. 2007; Wasilewska *et al*. 2008). In seeds ABA is necessary for the induction of dormancy, the induction of desiccation tolerance and the synthesis of proteins involved in seed maturation or for storage (Finkelstein *et al*. 2002). ABA has also been implicated in other dormancy processes such as axillary bud dormancy and apical bud dormancy (Olsen 2003; Ruttink *et al*. 2007). In apical bud dormancy ABA seems to have similar functions as in seed dormancy, and it was suggested that there are parallels in the underlying molecular mechanisms of

dormancy induction in seeds, cambial tissue and apical buds (Ruttink *et al*. 2007). During fruit development a function for ABA is mainly thought to be restricted to seed maturation (Gillaspy *et al*. 1993; Srivastava and Handa 2005). A function for ABA in fruit set has not been established. However, it was suggested that in pea ABA has an inhibiting effect on fruit set, and this inhibition was closely related to an interaction with GA and auxin. After decapitation of the shoot apical meristem parthenocarpic fruit set is often observed in pea. IAA transported basipetally from the apical shoot prevented this. The effect of IAA was indirect and thought to be mediated by ABA (Rodrigo and Garcia-Martinez 1998). In earlier work in pea it was already observed that ABA application could counteract the stimulating effect of GA on fruit set (Garcia-Martinez and Carbonell 1980). The transcriptome analysis of tomato ovaries described in Vriezen *et al*. (2008) also suggests a function for ABA in tomato fruit set.

Scope of the thesis

The research presented here was undertaken to gain more insight in the role of abscisic acid during fruit development in tomato, focussing on late ovary maturation and early fruit development. To complement the transcriptome analysis described in Vriezen *et al.* (2008) we examined the changes in ABA hormone levels and the expression of genes involved in ABA metabolism in mature unpollinated ovaries and after pollination (chapter two). Chapter two additionally describes the functional characterization of *SlCYP707A1,* a gene involved in ABA catabolism. Furthermore, we tried to obtain more information about the role of ABA in fruit development by using three different strategies. First, we used ABA-deficient mutants*, notabilis* and *flacca,* and a cross between these mutants, a *notabilis-flacca* double mutant, to study the effect of reduced ABA levels on fruit development (chapter three). Second and third we set out to develop ABA hypersensitive and ABA-insensitive plants respectively, by knocking down a *RPN10*-homologue and over-expressing and knocking-down an *ABI1*-homologue in tomato. These genes were chosen because they were shown to code for ABA-signaling components, and mutants in these genes have been described as ABA hypersensitive or ABA insensitive in Arabidopsis (Smalle *et al*. 2003; Koornneef *et al.* 1984; Merlot *et al.* 2001). Additionally, it was shown that the *ABI1*-homologue was differentially expressed after pollination, suggesting a function in fruit set (Vriezen *et al.* 2008). Both strategies were intended to result in the tools by which we could examine the effect of changed ABA-sensitivity on the process of early fruit development (chapter four and five). Finally we studied a tomato homologue of a gene that has been described as a dormancy

marker in pea. Since it is associated with the dormancy-like state and it is possibly regulated by auxin, gibberellin and ABA (Park and Han 2003), it may shed light on the state of dormancy in the mature unpollinated ovary. Studies on the expression, regulation and function of this gene are presented in chapter six. The results of the research are discussed and placed in wider perspective in the general discussion, chapter seven.

Chapter 2

Abscisic acid levels in tomato ovaries are regulated by *LeNCED1* and *SlCYP707A1*

L. Nitsch, C. Oplaat, R. Feron, Q. Ma, M. Wolters-Arts, P. Hedden, C. Mariani, W. H. Vriezen Planta 2009

Abstract

Although the hormones gibberellin and auxin are known to play a role in the initiation of fruits, no such function has yet been demonstrated for abscisic acid (ABA). However, ABA signaling and ABA responses are high in tomato (*Solanum lycopersicon* L.) ovaries before pollination and decrease thereafter (Vriezen *et al.* 2008). As a first step to understanding the role of ABA in ovary development and fruit set in tomato we analyzed ABA content and the expression of genes involved in its metabolism in relation to pollination. We show that ABA levels are relatively high in mature ovaries and decrease directly after pollination, probably caused by a decrease in ABA biosynthesis and increase of ABA catabolism. An important regulator of ABA biosynthesis in tomato is 9-*cis*-epoxycarotenoid dioxygenase (LeNCED1), whose mRNA level in ovaries reduced after pollination. The increased catabolism is likely caused by strong induction of one of four newly identified putative (+)ABA 8`-hydroxylase genes. This gene was named *SlCYP707A1* and is expressed specifically in ovules and placenta. Transgenic plants, over expressing *SlCYP707A1,* have reduced ABA levels and exhibit ABA-deficient phenotypes suggesting that this gene encodes a functional ABA 8`-hydroxylase. Gibberellin and auxin application have different effects on the *LeNCED1* and *SlCYP707A1* gene expression. The crosstalk between auxins, gibberellins and abscisic acid during fruit set is discussed.

Introduction

A major and nutritionally important part of the human diet is composed of fruits or fruit-derived products. Additionally, fruits are important in the life-cycle of plants, since they protect the seeds during their development and allow seed dispersal. Therefore, there has been considerable research on fruit development, for which tomato has been used as a model system for physiological and molecular studies for a long time. The first step of fruit development, fruit initiation, is very important from an agro-economical point of view. It is a very delicate phase, which is sensitive to abiotic factors and these often decrease fruit set and thus reduce yields. From a developmental point of view, fruit initiation, commonly referred to as fruit set, is the stage at which the ovary makes the decision to abort or to proceed with further cell division and fruit development (Gillaspy *et al.* 1993). Normally, fruit set is dependent on successful completion of pollination and fertilization (Gillaspy *et al.* 1993). However, it is not known precisely how the signals from pollination and fertilization stimulate cell division and fruit initiation.

It has long been known that application of hormones such as auxins or gibberellins can induce fruit set in the absence of fertilization. The resulting parthenocarpic fruit growth is a clear demonstration of the importance of hormones in fruit initiation and development. There are a number of parthenocarpic tomato mutants, such as *pat*, *pat2* and *pat3/4*, in which it was shown that the gibberellin content and/or gibberellin biosynthesis were increased significantly (Mazzucato *et al.* 1998; Fos *et al.* 2000, 2001; Olimpieri *et al.* 2007). Furthermore, over-expression of the *iaaM* gene, which encodes an enzyme involved in auxin biosynthesis, also induced parthenocarpic fruit development in tomato and many other species (Rotino *et al.* 1997; Mezzetti *et al.* 2004). Changes in both gibberellin and auxin content were thus associated with parthenocarpic phenotypes. The altered hormone balance in the ovary of parthenocarpic plants seems therefore to substitute for pollination and fertilization (Gorguet *et al.* 2005).

Some of the genes functioning in the biosynthesis and signaling cascade of these hormones during fruit initiation have been identified recently. For instance, it was shown that expression of a GA 20-oxidase gene was induced by pollination in tomato (Rebers *et al.* 1999; Serrani *et al.* 2007) and, the importance of SlDELLA, which represses GA-signaling and tomato fruit set, has also been demonstrated (Marti *et al.* 2007). Additionally, IAA9 and ARF8, both transcriptional regulators of the auxin response, were shown to be part of an important signal for fruit set (Wang *et al.* 2005; Goetz *et al.* 2007). However, many other genes remain to be discovered and investigated. We have used transcript profiling to gain a better insight into the genes involved in fruit initiation, focusing on genes involved in hormonal signaling. Several groups of genes were found to have altered mRNA levels after pollination including genes involved in the cell cycle, posttranscriptional gene regulation, GA-biosynthesis and auxin signaling (Vriezen *et al.* 2008). Notably, several genes related to abscisic acid (ABA), such as *ABA RESPONSE ELEMENT BINDING PROTEIN 1*, *ABA INSENSITIVE1*-like genes, and dehydrin genes, were found to be highly expressed in mature ovaries while their expression decreased after pollination (Vriezen *et al.* 2008). Many factors related to ABA signaling and ABA responses are thus higher in mature, unpollinated ovaries and to be quickly reduced after pollination. Abscisic acid is known to play a role in seed and bud dormancy, and in the regulation of abiotic stress responses (Horvath *et al.* 2003; Bartels and Sunkar 2005; Finkelstein *et al.* 2008). During tomato fruit development, its role is thought to be primarily restricted to the latter half of seed development in processes such as reserve deposition, desiccation tolerance and seed dormancy (Gillaspy *et al.* 1993); a role in the first phase of fruit initiation has not been established. ABA has, however, been mentioned

as a player in pea fruit set (Garcia-Martinez and Carbonell 1980; Rodrigo and Garcia-Martinez 1998). Here we describe the changes found in the ABA content of tomato ovaries and the regulation of ABA biosynthesis and catabolism genes during fruit initiation. Our data suggest that ABA is an additional player in the regulation of tomato fruit set together with auxin and gibberellin.

Results

ABA content

ABA signaling and ABA response genes are highly expressed in unpollinated ovaries and their expression decreases upon pollination (Vriezen *et al.* 2008), suggesting a concomitant decrease in ABA concentration. To confirm this assumption ABA concentration was determined in ovaries at several time points before and after pollination. Figure 1 shows that the free-ABA level in ovaries significantly decreased from 6.7 ng/mg DW before pollination (C0), to 2.4 ng/mg DW three days after pollination (dap). In contrast, if ovaries were kept unpollinated during the same period, the free-ABA level increased to 7.6 ng/mg DW (C3).

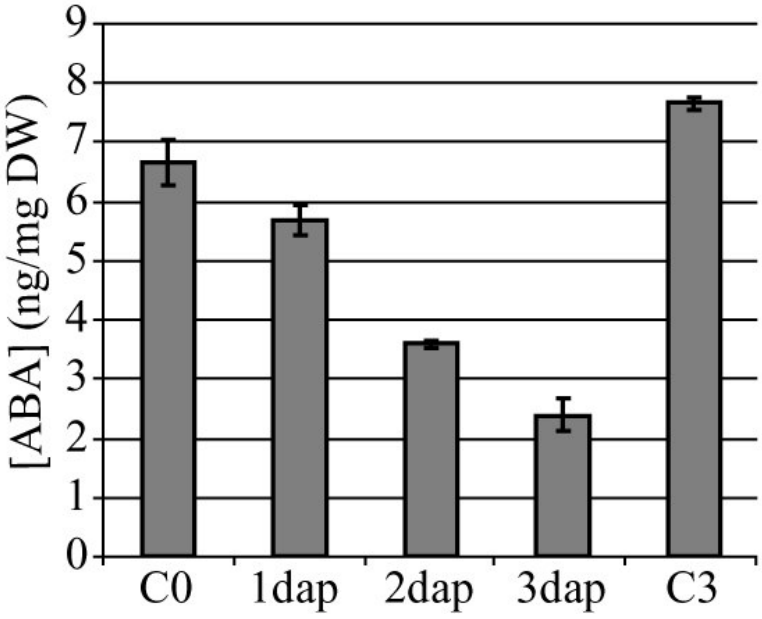

Fig. 1: ABA concentration decreases in ovaries after pollination. ABA concentration in unpollinated ovaries of emasculated flowers at full bloom (C0) and in unpollinated ovaries three days after full bloom (C3) and, 1-3 dap: one, two, and three days after pollination. Mean values (n= 3) ± SE are depicted.

Regulation of ABA biosynthesis

It is generally accepted that the cleavage reaction catalyzed by 9-*cis*-epoxycarotenoid dioxygenases (NCED) is a major rate-limiting step and point of regulation in ABA biosynthesis (Qin and Zeevaart 2002).We demonstrated that *LeNCED1* gene expression was significantly down-regulated in the ovary after pollination. In other species, like Arabidopsis and rice, NCED enzymes are encoded by small gene families composed of 5 and 3 members respectively (Tan *et al.* 2003). However, in tomato only one NCED (*LeNCED1*) has been identified so far, and shown to have 9-*cis*-epoxycarotenoid cleavage activity. In an attempt to obtain a complete overview of the transcriptional regulation of the whole NCED gene family in tomato, we searched the DFCI-Tomato Gene Index and NCBI EST databases for sequences homologous to *LeNCED1* or to one of the five known Arabidopsis genes. The neighbor joining tree in figure 2 shows that the four tomato sequences most similar to *LeNCED1* did not group together with the *LeNCED1* and the other functional NCED genes from Arabidopsis, rice, potato and maize.

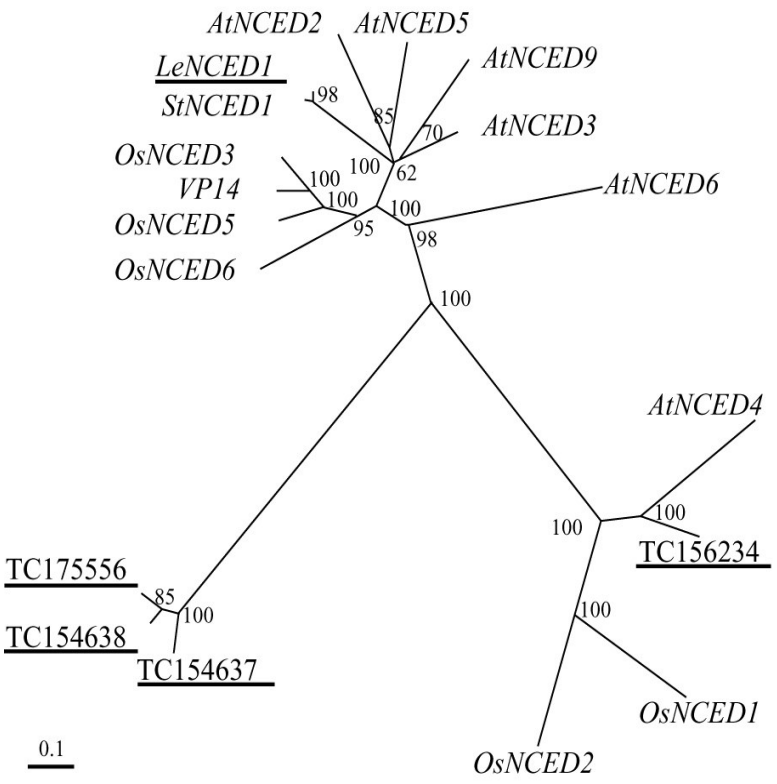

Fig. 2: Neighbor joining tree of NCED and NCED-like sequences with bootstrap values indicated at junctions. *LeNCED1* groups together with known NCED sequences such as *AtNCED2,3,5,6,9, VP14* and *OsNCED3,5,6.* Two other groups are formed including TC175556, TC154638, TC154637, TC156234, *OsNCED1,2* and *AtNCED4*. Tomato sequences are under-lined. For accession details see Materials and methods.

Three of the four tomato sequences (TC175556, TC154638, and TC154637) formed a separate group, while the other sequence (TC156234) grouped together with other carotenoid cleavage dioxygenases (CCD's) that are not known to be related to ABA biosynthesis (Tan *et al.* 2003). Additionally, the deduced amino acid sequences of the four tomato sequences were less than 35 percent identical to the functional Arabidopsis or tomato NCED amino acid sequences. Therefore we think it is possible that no additional functional NCED genes are present in the tomato genome.

LeNCED1 mRNA levels decreased after pollination in both the ovules and placenta tissue, and the pericarp (Fig. 3A). Semi-quantitative RT-PCR analysis, with specific *LeNCED1* primers, revealed that it is expressed equally in wall, ovules and placenta in unpollinated ovaries (Fig. 4A). After pollination there is a decrease in expression in wall and ovules. Because of its equal distribution throughout the unpollinated ovary no *in situ* hybridization was performed for *LeNCED1*.

Regulation of ABA catabolism

ABA concentration is regulated by catabolism as well as synthesis. In plant cells ABA is mainly inactivated by 8`-hydroxylation (Nambara and Marion-Poll 2005), which in Arabidopsis, is catalyzed by four cytochrome P450 mono-oxygenases, *AtCYP707A1* to *AtCYP707A4* (Kushiro *et al.* 2004). We previously identified a tomato homolog of *AtCYP707A4*, which was strongly induced in the ovary after pollination (Vriezen *et al.* 2008). We now isolated and characterized the corresponding full length (1742bp) cDNA clone from a tomato ovary cDNA-library. The deduced amino acid sequence is highly homologous to the Arabidopsis CYP707A4 protein (65% amino acid identity) and *Solanum tuberosum* CYP707A2 gene (95% identity). The tomato protein (designated SlCYP707A1) contains the highly conserved cysteine residue (within the PFGNGTHS**C**PG motif), which is the putative heme-iron-ligand, common to all P450s and essential for catalytic activity (Kushiro *et al.* 2004). A database search yielded three more putative ESTs (TC177455, AI484420, and TC186477) that share between 61% to 68% sequence identity with *SlCYP707A1* at nucleotide level. Their deduced protein sequences are each 70% to 74% identical with one of the Arabidopsis proteins. We therefore consider the three genes to encode putative ABA 8`-hydroxylases, and designated them *SlCYP707A2*, *SlCYP707A3* and *SlCYP707A4*.

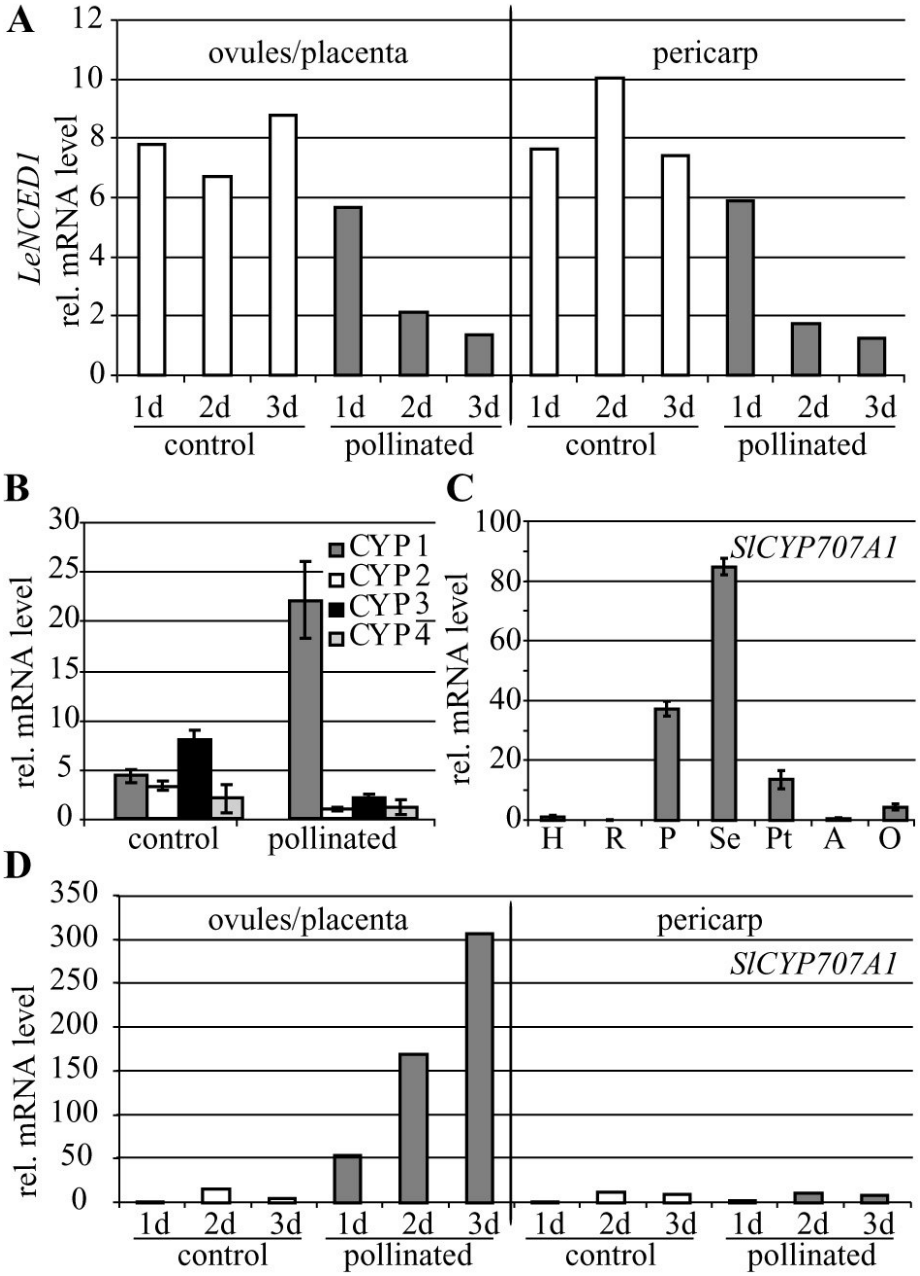

Fig. 3: A) Relative mRNA levels of LeNCED1. LeNCED1 mRNA levels decrease in both pericarp and ovules/placenta after pollination. **B)** mRNA levels of SlCYP707A1, SlCYP707A2, SlCYP707A3, and SlCYP707A4 (CYP1 - CYP4) in ovaries 3 days after full bloom (control) or after pollination. **C)** mRNA levels of SlCYP707A1 in different generative and vegetative tissues of tomato; hypocotyl (H) and root (R) tissue from 10 day old seedlings, and pedicel (P), sepal (Se), petal (Pt), anther (A) and ovary (O) tissue from flowers 3 days before full bloom. **D)** mRNA levels of SlCYP707A1 in more detail in separated ovules/placenta and pericarp tissue. SlCYP707A1 mRNA levels are increased after pollination specifically in ovule/placenta tissue. Mean values (n= 4) ± SE are depicted.

The mRNA levels of the four putative tomato *CYP707A*-genes were determined in emasculated flowers three days after full bloom (control) and three days after pollination in tomato ovaries. Figure 3B shows that the mRNA concentration of *SlCYP707A1* strongly increased after pollination in whole ovaries and it reached a much higher level than the mRNA levels of the other *CYP707A*-genes. Additionally, the mRNA levels of all other putative tomato *CYP707A*-genes were reduced after pollination. This suggests that *SlCYP707A1* is the most important gene responsible for ABA catabolism in pollinated ovaries. *SlCYP707A1* expression was also analyzed in other tissues. Figure 3C demonstrates that it is also expressed in sepals, petals and pedicels. The accumulation of *SlCYP707A1* mRNA in ovaries was determined more precisely in separated ovule/placenta and pericarp tissue during the first three days after pollination. *SlCYP707A1* mRNA levels were several hundredfold up-regulated after pollination, specifically in ovules/placenta and not in pericarp (Fig. 3D).

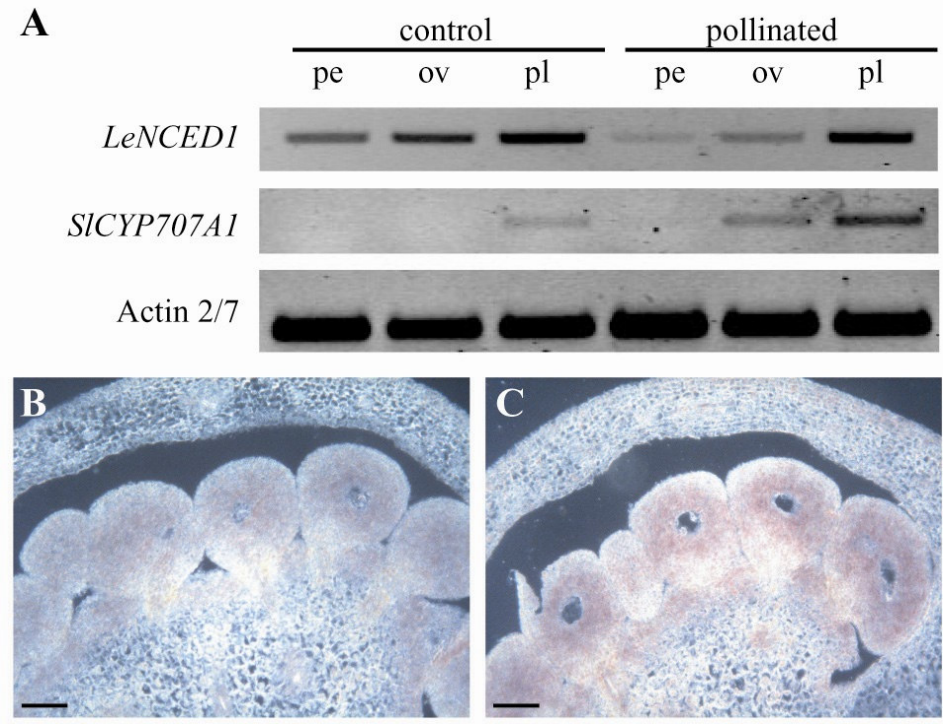

Fig. 4: Localization of LeNCED1 and SlCYP707A1 mRNA in ovaries. **A)** Semi-quantitative RT-PCR with LeNCED1 and SlCYP707A1 specific primers, and actin 2/7 as an internal control gene. LeNCED1 is equally expressed in wall, placenta and ovules of unpollinated ovaries and expressed lower after pollination in wall and ovules. SlCYP707A1 is weakly expressed in the placenta of unpollinated ovaries and expressed higher in both ovules and placenta in pollinated ovaries. In situ hybridization with a sense **(B)** and anti-sense **(C)** RNA probe of SlCYP707A1. Alkaline phosphatase activity gives a purple staining. Bar represents 100 µm

Furthermore, mRNA localization was analyzed in ovules and placenta separately. In unpollinated (control) ovaries *SlCYP707A1* mRNA is present in the placenta, but not in the ovules and pericarp (Fig. 4A). In pollinated ovaries *SLCYP707A1* mRNA level is generally higher and present in both placenta and ovule tissue. *In situ* hybridization with a gene-specific probe confirmed this localization of *SlCYP707A1* in pollinated ovaries. The purple staining suggests relatively high *SlCYP707A1* mRNA levels in the ovules and in the periphery of the placenta but low levels in the pericarp (Fig. 4B,C).

Gene-expression data suggest that the reduction in ABA levels that we measured after pollination could be due to increased ABA 8`-hydroxylation activity. To strengthen this hypothesis we analyzed the levels of the ABA catabolic products in ovaries three days after pollination and three days after anthesis in unpollinated ovaries. Table 1 shows that the level of ABA in unpollinated ovaries was two-and-half-fold higher than in pollinated ovaries, comparable to the measurements shown in figure 1. The product of ABA 8`-hydroxylation is (-)-phaseic acid (PA) which can be converted to (-)-dihydrophaseic acid (DPA). PA levels did not change, but DPA levels increased three-fold in ovaries after pollination. 7`- and 9`-ABA hydroxylation can also inactivate ABA in several plant species (Nambara and Marion-Poll 2005). The product of ABA 9`-hydoxylation is 9`-hydroxy ABA which is converted to neo-phaseic acid (neo-PA). In tomato ovaries a considerable amount of neo-PA was present, but this was not affected by pollination. The presence of 7`-hydroxy ABA was also detected, but its level was low and could not be quantified significantly. The amount of the conjugated form of ABA, ABA-glucosyl ester (ABA-GE), in unpollinated ovaries was twice as high as that in pollinated ovaries (Table 1).

Table 1. ABA and catabolite levels in unpollinated and pollinated ovaries

	ABA (ng/mg DW)	PA (ng/mg DW)	DPA (ng/mg DW)	ABA-GE (ng/mg DW)	neo-PA (ng/mg DW)
unpollinated	10.76 (± 0.12)	0.10 (± 0.05)	0.44 (± 0.05)	1.32 (± 0.04)	0.46 (± 0.09)
Pollinated	4.26 (± 0.31)	0.10 (± 0.06)	1.20 (± 0.08)	0.65 (± 0.05)	0.46 (± 0.01)

Mean values (*n*=2) ±SE are depicted

Functional analysis of SlCYP707A1

To prove that the *SlCYP707A1* gene is coding for a functional ABA-8`-hydroxylase, we over-expressed its coding region under control of the 35S-promoter in tomato. Several transgenic lines were obtained, of which the line with the highest over-expression level was further analyzed. Figure 5A shows the *SlCYP707A1* mRNA levels in unpollinated mature ovaries. The *SlCYP707A1* over-expression line has approximately 45-fold higher mRNA levels than wild type. Consequently, the free-ABA level in unpollinated ovaries was 46% lower in *SlCYP707A1* over-expression plants than in wild type plants (Fig. 5B), confirming the ABA 8`-hydroxylase activity of *SlCYP707A1*. The over-expression plants have a smaller leaf surface area and wilt stronger 72 hours after water with holding than wild type plants (Fig. 5C, D). In addition, we also observed the initiation of adventitious root growth on the stem of the *SlCYP707A1* over-expression plants (Fig. 5E).

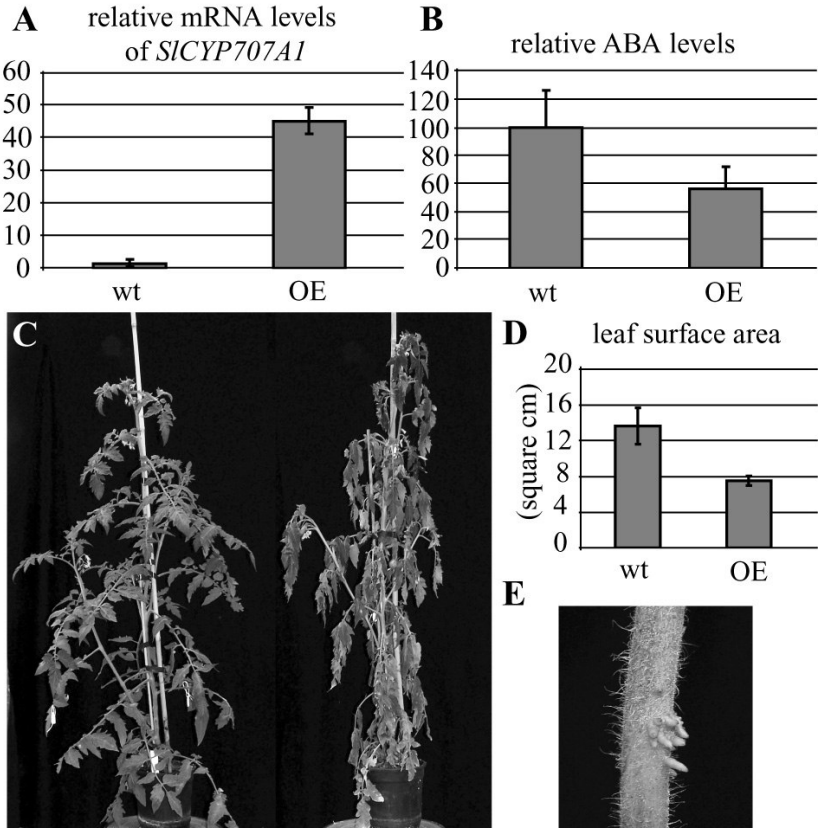

Fig. 5: A) Relative mRNA levels of the *SlCYP707A1* gene in unpollinated mature ovaries of over-expression (OE) plants are 45 times higher than wild type (wt). **B)** Relative ABA levels in unpollinated mature ovaries of over-expression plants is 56% of wild type. **C)** Over-expression plants (right) wilt stronger after 72hours of water with holding than wild type plants (left). **D)** The leaf surface area of the compound leaves of over-expression plants is smaller than wild type. **E)** On the stem of the over-expression plants small adventitious roots are visible.

Hormone crosstalk

Because fruit set is induced by gibberellin and auxin, we were interested in the effect of these hormones on the activity of both, the *LeNCED1* gene and the *SlCYP707A1* gene. Figure 6A shows that the *LeNCED1* mRNA level in ovaries was lower after GA_3 treatment or 4-CL-IAA treatment and comparable to the levels found after pollination. ABA treatment however increased the mRNA level of *LeNCED1*. Figure 6B shows that *SlCYP707A1* mRNA level was not affected by GA_3 treatment in contrast to pollination, 4-Cl-IAA treatment, or ABA treatment, which all had a stimulating effect.

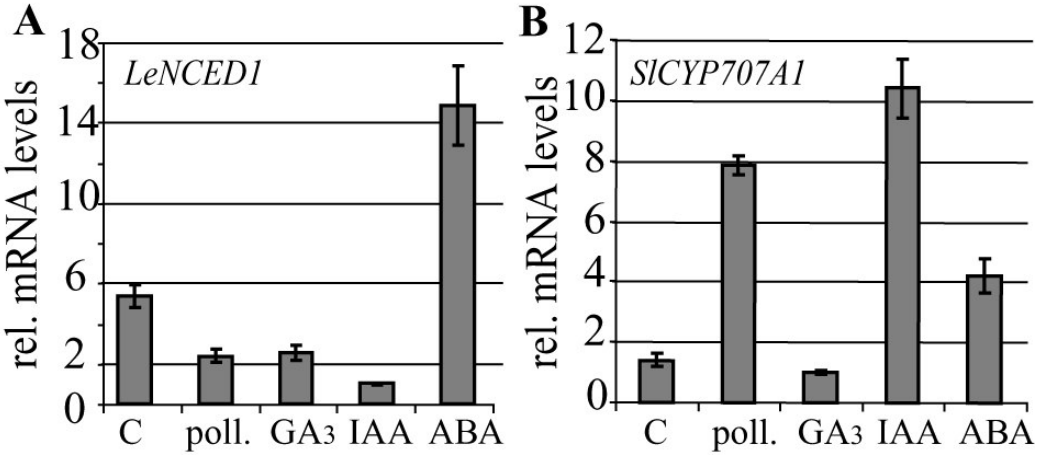

Fig. 6: mRNA levels of *LeNCED1* and *SlCYP707A1* before (C=control) and after hormone treatment or after pollination. **A)** *LeNCED1* expression is induced by ABA treatment and reduced after pollination, GA_3 and 4-Cl-IAA (IAA) treatment. **B)** *SlCYP707A1* expression is induced after pollination, 4-Cl-IAA and ABA treatment. Mean values (n= 6) ± SE are depicted.

Discussion

ABA content

Fruit set is an agro-economically important process, in which hormones play a pivotal role. Gibberellins and auxins were known previously to induce fruit growth and, by demonstrating that ABA signaling is high in mature ovaries and decreases after pollination, we have recently suggested a role for ABA in fruit set (Vriezen *et al.* 2008). Additionally, ABA has been immuno-localized in the ovary of Arabidopsis and cucumber, indicating a function for ABA in these tissues (Peng *et al.* 2006). Previously, Kojima *et al.* (1993) had measured relatively high levels of ABA in unpollinated pistils compared to 10- day-old tomato fruits. Here we have demonstrated that within three days the free ABA content of pollinated tomato ovaries decreases to approximately 30% of that in unpollinated ovaries. A

decrease in ABA concentration to 50% or 25% of the original concentration has also been measured in other physiological systems, such as in dormancy breakage in seeds (Chiwocha *et al.* 2005) and tubers (Destefano-Beltran *et al.* 2006). The relative decrease in ABA content in the tomato ovary is comparable to those observed in other processes in which ABA level is of physiological importance.

Regulation of ABA biosynthesis and catabolism

ABA concentration is actively regulated in ovaries, as we found biosynthesis genes to be down-regulated and ABA catabolism genes to be induced in the ovary after fruit initiation. NCED genes are often encoded by a small gene family. However, we were not able to find putative NCED genes other than *LeNCED1* in the tomato sequence databases, which contain 213947 ESTs and 41425 unique sequences. The three other CCDs described are probably not related to ABA biosynthesis as they do not group with *LeNCED1*. Moreover, TC154637 and TC154638 have already been described as *carotenoid cleavage dioxygenase 1* genes involved in volatile terpenoid production (Simkin *et al.* 2004). Therefore, *LeNCED1* appears to produce the most represented NCED transcript in ovary and possibly is the only *NCED* gene in tomato. In addition, the fact that the *notabilis* mutant with a mutation in the *LeNCED1* gene has a strong ABA deficient phenotype (Burbidge *et al.* 1999; Thompson *et al.* 2004), indicates that this gene has a prominent role in ABA biosynthesis. Transcript levels for this gene decreased after pollination concomitant with the decrease in ABA concentration, indicating that *LeNCED1* regulation directly affects biosynthesis of ABA in the mature ovary.

Transcription levels of a gene very homologous to an Arabidopsis ABA 8`-hydroxylase (*AtCYP707A4*) were strongly induced specifically in ovules and placenta tissue, in contrast to the mRNA levels of the three other putative tomato *CYP707A*-genes. Similarly, the *CYP707A*-genes in Arabidopsis are also differentially expressed, for instance during dehydration and rehydration responses and during seed dormancy (Kushiro *et al.* 2004; Okamoto *et al.* 2006). The deduced protein sequence of *SlCYP707A1* has very high homology to *AtCYP707A4.* Moreover, we indirectly proved that the *SlCYP707A1* gene is encoding for a functional ABA 8`-hydroxylase by over-expressing it in tomato. This resulted in reduced ABA levels and ABA deficient phenotypes, such as adventitious rooting, reduced leaf surface area and increased wilting. These characteristics have also been described for the tomato ABA-deficient mutants, *sitiens*, *notabilis* and *flacca* (Taylor and Tarr 1984; Burbidge *et al.* 1999). The remarkably large increase we found in *SlCYP707A1* mRNA levels suggests a strong induction of ABA 8`- hydroxylation after pollination. Additionally, we showed that

after pollination a higher level of dihydrophaseic acid (DPA), the end product of the ABA 8`-hydroxylation pathway, was present in the ovary, indicating that ABA 8'-hydroxylation was induced after pollination. Interestingly, also 7`- and 9`-hydroxylation of ABA takes place in tomato. However, no differences were found between 7`-hydroxy ABA and neo-PA levels in ovaries after pollination, suggesting that these conversions take place, but do not attribute much to the decrease in ABA levels after pollination. Apparently, ABA 8`-hydroxylation is the major ABA catabolic pathway in tomato ovaries (our work) as was suggested for other species (Nambara and Marion-poll 2005). ABA can be transported and stored as ABA-GE (Nambara and Marion-poll 2005) and the high levels in unpollinated ovaries could mean that ABA-GE is transported to, and stored in the ovary. Altogether these data strengthen our hypothesis that after pollination ABA levels are reduced mainly through ABA 8'-hydroxylation. The specific localization of *SlCYP707A1* in ovules and placenta might indicate that embryo or seed formation requires rapid removal of ABA. The localization of *SlCYP707A1* mRNA is comparable to the localization of the hormone ABA in the ovules of Arabidopsis and cucumber (Peng *et al.* 2006). The decrease in mRNA levels of the three other putative tomato CYP707A genes might be explained by the reduction in ABA concentration, since positive feedback from ABA on CYP707A expression has been found in Arabidopsis (Kushiro *et al.* 2004; Umezawa *et al.* 2006), potato (Destefano-Beltran *et al.* 2006) and by us in tomato (Fig. 6B).

Hormone crosstalk

Auxin has been shown to interact with several hormones via regulation of expression of genes encoding biosynthetic or catabolic enzymes (Ross and O'Neill 2001). A fertilization-induced increase in auxin signaling most likely regulates the expression of ABA biosynthesis and catabolism genes during tomato fruit set, since we could show that auxin treatment reduced *LeNCED1* transcript levels and increased *SlCYP707A1* transcript levels. GA_3 treatment also reduced *LeNCED1* gene expression but it had no effect on *SlCYP707A1* gene expression. Therefore, auxin, via GA, or both hormones together regulate *LeNCED1* gene expression, whereas *SlCYP707A1* expression seems to be regulated by auxin alone. This would be consistent with auxin inducing GA biosynthesis, but not *vice versa*. It seems likely that a pollination-induced auxin increase precedes the gibberellin increase, as summarized in figure 7. This hormonal signaling cascade may also lead to a reduction in the ABA content via changes in *LeNCED1* and *SlCYP707A1* expression. In the promoter of *LeNCED1,* auxin, gibberellin, and ABA response elements were identified (Thompson *et al.* 2004), consistent

with *LeNCED1* being regulated by all three hormones. Since we found that *LeNCED1* is positively regulated by ABA, a decrease of ABA content after pollination might also contribute to the decrease in its mRNA levels. A positive feedback of ABA levels on *NCED* mRNA levels has been shown in Arabidopsis (Wan and Li 2006). However, Thompson *et al.* (2000) showed that ABA had no effect on *LeNCED1* expression in a detached leaf assay. Different tissues might thus have different regulatory mechanisms of *LeNCED1* expression. The increase in *SlCYP707A1* levels cannot be caused by a lower content of ABA, because this gene is also positively regulated by ABA.

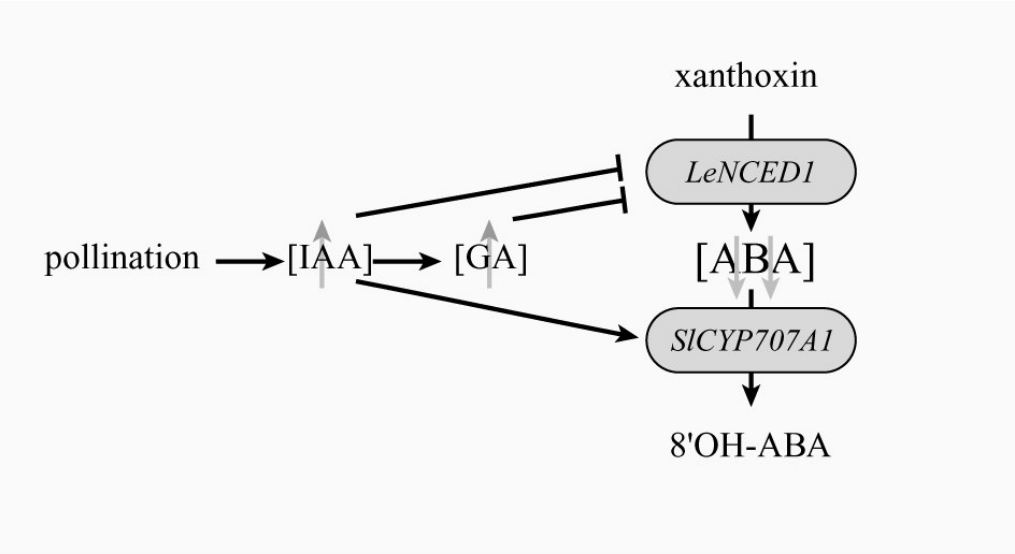

Fig. 7: Model of the hormonal interactions after pollination. Pollination induces an increase in auxin concentration and subsequently an increase in GA concentration. Together the increase in GA and IAA-levels inhibit the expression of the *LeNCED1* gene (ABA biosynthesis), while only increased IAA-levels can stimulate the expression of the *SlCYP707A1* gene (catabolism), ABA levels therefore decrease.

Function of ABA during fruit set

In a mature unpollinated ovary many cell cycle genes, such as *LeCDKB1,2*, *CycA1,2* and *CycD3* are relative lowly expressed (Vriezen *et al.* 2008). In addition, the relative expression level in the mature tomato ovary of two genes homologous to *PsDRM1*, which proved to be excellent dormancy (DRM) markers in pea (Stafstrom *et al.* 1998), are high. Together this suggests that the mature ovary before pollination has entered a temporarily dormant state, during which the tissue is quiescent. It is known that ABA can directly influence the cell cycle via suppression of *CDKA1* expression (Smalle *et al.* 2003) or stimulation of the *ICK1* (an inhibitor of CDK action) expression (Wang *et al.* 1998), thereby inhibiting cell division and inducing an quiescent state. We know that the ABA-signaling and

response pathway are active in the unpollinated mature ovary (Vriezen *et al.* 2008). Additionally in the research presented here we have shown that ABA levels are relatively high in mature ovaries and are down regulated after pollination. Based on these observations a hypothesis is that ABA inhibits growth until fruit set. The post pollination increase of auxin and gibberellin levels may directly or indirectly repress ABA biosynthesis. However, we were unable to induce fruit set by application of an ABA-biosynthesis inhibitor, fluridone. Neither could we inhibit fruit set by ABA application to pollinated ovaries (data not shown). This might indicate that not only the concentration of ABA but rather the hormonal balance of ABA and other hormones, such as auxin and gibberellin, is important. Similarly, the potential of axillary bud outgrowth is determined by a balance of the hormones cytokinin, auxin and ABA (Shimizu-Sato and Mori 2001). Besides inhibition of growth, ABA can also induce tolerance to several abiotic stresses, such as heat stress, cold stress and drought stress (Bartels and Sunkar 2005; Gusta *et al.* 2005; Bonham-Smith *et al.* 2007). For example, ABA induces dehydrins which are proteins with a potential *in vivo* role in stabilizing cells under abiotic stress (Kalemba *et al.* 2007). Several dehydrins are indeed higher expressed in the mature unpollinated ovary (Vriezen *et al.* 2008). A putative protective function for ABA might become more apparent during unfavorable environmental conditions when ABA protects the mature ovary during its temporal dormant state.

Acknowledgements

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Materials and methods

Plant material

Tomato plants (*Solanum lycopersicum* L. cv. Moneymaker from Enza Zaden, Enkhuizen, the Netherlands) were grown under greenhouse conditions from March to October under a 16/8h day-night rhythm. Supplementary lights (600 Watt high pressure sodium lights) turned on below 200 W/m^2 and turned off above 300 W/m^2. Temperature was kept above

20°C during the light period and 17°C during the dark period with the PRIVA Integro versie 724 system. Plants were watered daily and given fertilizer weekly.

Treatments

Flowers were emasculated 3 days before full bloom (dbf) and at full bloom either hand pollinated or treated with 2 µL of either 1 m*M* GA_3 (Duchefa Biochemie BV, Haarlem, the Netherlands) in 1% (v/v) ethanol, or 1 m*M* 4-Cl-IAA (Sigma-Aldrich, St. Louis, MO, USA) in 2% (v/v) ethanol, or 1 m*M* ABA (Acros, Geel, Belgium) in 10% (v/v) methanol. 4-Cl-IAA is a more stable form of auxin than IAA and was used because it appeared to induce tomato fruit set more reliably than IAA. Control flowers were treated with 2 µL of the solvent (10% methanol or 1% ethanol). Hormone treatments were repeated after two days. Flowers were harvested three days after first treatment unless otherwise noted. Plant material was sampled between 11.00h and 13.00h and directly frozen in liquid nitrogen. When necessary, plant material was dissected using binoculars and frozen thereafter in liquid nitrogen. The different tissues were dissected from young flowers that were about 3-dbf. Roots and hypocotyls were isolated from 10-day-old seedlings.

ABA analysis

ABA was quantified in triplicates by GC-MS as described for GA analysis (Griffiths *et al.* 2006), but with modifications. Freeze-dried samples were homogenized in 50 ml 80% (v/v) methanol/water in 100 mL-flasks after which 300 ng 3-methyl-[$^2H_3$]ABA was added as internal standard. Samples were purified as described previously except that the pooled ethyl acetate phases after elution from the Varian Bond Elut NH_2 cartridge (100 mg; Kinesis, St. Neots, UK) were evaporated to dryness *in vacuo*, and dissolved in ethyl acetate (20 µL) of which 2 µL was injected into a TR-1 capillary column (30 m x 0.25 mm x 0.25 mm film thickness; Thermo Fisher Scientific, Madison, USA) at 50 ºC. The split valve (50:1) was opened after 2 min and the temperature increased at 20ºC/min to 180ºC and then at 4ºC/min to 300ºC. The instrument was operated in selective ion monitoring mode, monitoring the ions at *m/z* 190 and 162 for ABA and 193 and 166 for [$^2H_3$]ABA. The amount of ABA was determined from the peak areas for the ions *m/z* 190 and 193 by reference to a calibration curve.

Neighbor joining tree

NCED-mRNA sequences from Arabidopsis, rice, maize, tomato and potato were obtained from NCBI (www.ncbi.nlm.nih.gov) or Dana-Farber Cancer Institute (DFCI) Tomato Gene Index (http://compbio.dfci.harvard.edu/tgi). The sequences were translated to protein and aligned with ClustalW (www.ebi.ac.uk/clustalw/). A neighbor joining tree was generated using the PHYLIP Protdist program (Phylogeny Inference Package version 3.5c; Felsenstein, J. Department of Genetics, University of Washington, Seattle) available on http://bioweb.pasteur.fr/seqanal/phylogeny/phylip-uk.html. Default parameters were used and 100 Bootstrap replicates were generated. The consensus tree was drawn in TreeView (free available from taxonomy.zoology.gla.ac.uk /rod/treeview.html). An unrooted tree was drawn from output data without bootstrap values, bootstrap values (calculated by Prodist) were manually added afterwards.

Isolation and cloning of *Sl*CYP707A1 and CYP707 like cDNAs

The full length *SlCYP707A1* sequence was isolated by plaque screening of a phage cDNA library (HybriZAP® 2.1, Stratagene, La Jolla, CA, USA) with a probe corresponding to base 1024 to 1387 of *Sl*CYP707A1, following the manufacturer's protocol. TC177455, AI484420 and TC186477 sequences were obtained from the DFCI Tomato Gene Index.

RNA isolation, cDNA-synthesis and Q-PCR data analysis

RNA was isolated with the RNAeasy kit (Qiagen, Valencia, CA, USA). Photometric RNA measurements were done to equilibrate the RNA concentrations of different samples. Equal amounts of RNA were DNase treated (RQ1, Promega, Madison, WI, USA). RNA (0.5 µg) was reverse transcribed (RT) in a total volume of 10µL using a cDNA synthesis kit (iScript^{tm}, Bio-Rad Laboratories, Hercules, CA, USA) following manufacturer's protocol.

Real-time-quantitative RT-PCR (Q-PCR) primers were designed using a computer program (Beacon Designer Software, Premier Biosoft International, CA, USA). Alignments of genes that were part of a gene family were made and gene specific DNA pieces were used for primer design. Primer pairs are depicted in table 2. Specificity was tested by sequencing the amplicon. Q-PCRs were done using SYBR green mix (iQ-SYBR Green Supermix, Bio-Rad Laboratories). PCR reactions were performed in a 96-well thermocycler (Bio-Rad iCycler) using a temperature program starting with 3 min at 95°C then 40 cycles consisting of

15 s at 95°C and 45 s at 57°C and finally the melting temperature of the amplified product was determined to verify the presence of a single product. Five micro liter of 25-fold diluted cDNA was used per sample. Technical and biological replicates were always performed. Both Actin 2/7 and Ubiquitin 7 were used as internal control genes, to correct for difference in cDNA amounts. Diluted DNase treated RNA was also included in the Q-PCR as a control for genomic DNA contamination.

Relative mRNA levels were calculated following the Bio-Rad outlined methodology based on Vandesomple *et al.* (2002) and corrected for PCR efficiencies, which were determined using dilution ranges (0.1 pg/µL - 10 pg/µL). The average of two biological repeats and two technical repeats is depicted together with the SE. When error bars were omitted in graphs, biological replicates showed the same trend but the absolute amounts were different – in these cases only one biological replicate is shown.

Semi-quantitative RT-PCR

Semi-quantitative-PCRs were performed using 5 µL of 25-fold diluted cDNA, buffer IV, 2.5 m*M* $MgCl_2$, 0.5unit Red Hot Taq DNA polymerase (all from ABgene Limited, Epson, Surrey, UK), 0.4 mM dNTPs (Fermentas, St. Leon-Rot, Germany), and 0.1 µ*M* primers (Table 2) in a reaction of 30 cycles, each comprising of 15 s at 95°C (denaturation), 30 s at 57°C (primer annealing) and 10 s at 72°C (extension time).

Table 2. Primer sequences used for semi- and real-time quantitative RT-PCR

Gene name or number (annotation)	Primer sequences
LeNCED1 (9-cis-epoxycarotenoid dioxygenase)	5'-CTTATTTGGCTATCGCTGAACC-3'
	5'-CCTCCAACTTCAAACTCATTGC-3'
SlCYP707A1 (ABA-8'hydroxylase)	5'-AGAGAGGCTGTAGCTGAGTGG-3'
	5'-TTGGCAAGTTCATTCCCTGGAC-3'
TC177455 (ABA-8'hydroxylase)	5'-GCAATGAAAGCGAGGAAAGAGC-3'
	5'-TCGAGCTGCAAAGATGACTCC-3'
AI484420	5'-CTAAGGTGGCAAGGAGGAAGC-3'

(Cytochrome P450)	5'-GTGTCCTGGGCAGCAAAGAG-3'
TC186477 (ABA-8'hydroxylase)	5'-GAGCATTCAAACCCGAAGCC-3'
	5'-AATTGTACCCTGTTTCGAGCAC-3'
ACT 2/7 (actin 2/7)	5'-GGACTCTGGTGATGGTGTTAG-3'
	5'-CCGTTCAGCAGTAGTGGTG-3'
UBQ 7 (ubiquitin 7)	5'-CCCTGGCTGATTACAACATTC-3'
	5'-TGGTGTCAGTGGGTTCAATG-3'

In situ hybridization

The RNA probes were synthesized by transcribing bases 1467 to1648 of the *CYP707A1* cDNA using T7 (sense) and SP6 (anti sense) RNA polymerase and digoxigenin (DIG) labeled UTP (Roche Applied Science, Basel, Switzerland). DIG labeling efficiencies were tested by a spot assay. Tissue-fixation was done as described by Bereterbide *et al.* (2002). Embedded tissues were sliced into 8 µm sections. Paraplast Plus (Sigma-Aldrich, St. Louis, MO, USA) was removed with Histoclear (National Diagnostics, G, USA), sections were hydrated, immersed in TE (Tris-HCl pH7.5, 10 mM EDTA) and a proteinase K treatment was performed. Sections were then dehydrated again, and air dried for 2-3 h. The slides were incubated in a humidified box overnight at 50ºC, in 150 µL hybridization mixture (50% formamide, 0.5 µg/ml tRNA, 10% (w/v) dextran sulphate, 300 m*M* NaCl, 5 m*M* EDTA, 10 m*M* Tris-HCl pH 7.5) supplemented with 100 ng of probe. The next morning slides were immersed in a series of SSC (Na_3citrate, NaCl) washing buffers until 0.5x SSC. Anti-DIG-alkaline-phosphatase-coupled antibody (Roche Applied Science) was diluted 500-fold in 1% (w/v) BSA solution and after blocking a volume of 150 µL was added to the slides and incubated in a humid chamber at 37^{o}C for 1.5 h. After two washes the slides were put in color substrate reaction buffer (100 m*M* NaCl, 5 m*M* $MgCl_2$, 100 m*M* Tris-HCl pH 9.0, 10% (w/v) polyvinylalcohol, 0.24 mg/mL Levamisole (Sigma-Aldrich), 5 µL/ml NBT and 3.75 µL/ml BCIP (Roche Applied Science) in the dark until purple staining appeared. Staining was stopped by immersing the slides in TE.

ABA catabolite analysis

The ABA catabolite analysis was performed at the Plant Biotechnology Institute of the National Research Council of Canada (http://www.pbi.nrc.gc.ca/ENGLISH/technology-platforms/plant-hormone-profiling.htm) by high performance liquid chromatography electrospray tandem mass spectrometry (HPLC-ES-MS/MS) using deuterated internal standards, as described in Owen and Abrams (2009).

Over-expression lines

To generate transgenic over-expression *SlCYP707A1* lines, the coding region (base 6-1478) was PCR amplified and cloned in the Gateway entry vector pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA, USA). *SlCYP707A1* coding region was recombined between the Cauliflower Mosaic Virus 35S promoter in the pGD625 vector (Chalfun-Junior *et al.* 2005) and the *NOPALINE SYNTHASE* terminator. Transgenic plants were generated by *Agrobacterium tumefaciens*-mediated transformation. Cotyledons of 10-day-old seedlings were cut and pre-incubated overnight on co-cultivation MS-medium containing vitamin Gamborg B5, 1% (v/w) sucrose, 0.5 mg L^{-1} MES buffer, 0.8% (v/w) Daishin agar, 0.05 mg L^{-1} 2,4-D, 0.1 mg L^{-1} IAA, 2.0 mg L^{-1} zeatin, 200 μ*M* acetosyringone, pH 5.8. *Agrobacterium tumefaciens* (EHA 105) were grown to an OD600 of 1.0 and diluted 25 times in liquid LB media and grown for another 4 h to an OD600 between 0.2-0.3. Bacteria were pelleted and resuspended in liquid MS-medium containing vitamin Gamborg B5 mixture, 3% (w/v) sucrose, 0.5 mg L^{-1} MES buffer, 200 μ*M* acetosyringone. Cotelydones were incubated 30 min in the bacterial suspension, washed and placed back on the co-cultivation media. Two days after transformation cotyledons were placed on MS-medium containing vitamin Gamborg B5 mixture, 3% (v/w) sucrose, 0.5 mg L^{-1} MES buffer, 0.8% (v/w) Daishin agar, 0.1 mg L^{-1} IAA, 2.0 mg L^{-1} zeatin, 200 mg L^{-1} Cefotaxime and 50 mg L^{-1} Vancomycin and incubated for another two days. To induce callus and shoot formation cotyledons were transferred to shoot inducing MS-medium containing vitamin Gamborg B5 mixture, 1% (w/v) glucose, 0.5 mg L^{-1} MES buffer, 0.8% (w/v) Daishin agar, 0.1 mg L^{-1} IAA, 2.0 mg L^{-1} zeatin, 500 mg L^{-1} carbenicillin and 100 mg L^{-1} kanamycin. Cotyledons were transferred to fresh medium every 3 weeks. Elongated shoots of 2 to 4 cm were excized from the callus and transferred to rooting MS culture medium containing 1.5% (w/v) sucrose, 4% (w/v) purified agar (Oxoid Ltd, Hampshire, UK), 0.25 mg L^{-1} IBA, 100 mg L^{-1} Cefotaxime and 100 mg L^{-1} Vancomycin. Rooted plantlets were transferred to soil for further selection including, ploidy test and PCR

with primers specific for the kanamycin resistance gene. Media components and antibiotics were obtained from Duchefa Biochemie BV unless otherwise specified.

Water stress experiment

Pots of wild type and *SlCYP707A1* over-expression lines of the same age and size were saturated with water at the start of the experiment. Plants were withheld water from then on. Photographs were taken 72 h after start of the experiment.

Leaf surface area

Leaf surface area was measured for four compound leaves of 2 months-old tomato plants on a Li-3100 area meter (Li-Cor, Lincoln, NE, USA). Average leaf surface is depicted with SD. Leaves were always picked at the same height.

Accession details

GenBank accession numbers: *LeNCED1* (Z97215), *AtNCED2* (NM117945), *AtNCED3* (NM112304), *AtNCED4* (NM118036), *AtNCED5* (NM102749), *AtNCED6* (NM113327), *AtNCED9* (NM106486), *StNCED1* (AY662343), *OsNCED1* (AY838897), *OSNCED2* (AY838898), *OsNCED3* (AY838899), *OsNCED4* (AY838900), *OsNCED5* (AY838901), *VP14* (ZMU95953), *AhNCED1* (AJ574819), *SlCYP707A1* (EU183406), *SlCYP707A3* (AI484420), *StCYP707A2* (DQ206631), *AtCYP707A1* (NM202845) *AtCYP707A2* (NM128466), *AtCYP707A3* (NM180805), *AtCYP707A4* (NM112814), ACT2/7 (BT013707), UBQ7 (AK246454).
DFCI-Tomato Gene Index accession numbers: *TC175556, TC154638, TC154637, TC156234, SlCYP707A2* (TC177455), *SlCYP707A4* (TC186477).

Chapter 3

Vegetative and generative development of the tomato ABA-deficient *notabilis* and *flacca* mutants and the *notabilis-flacca* double mutant

L. Nitsch, C. Oplaat, P. Michieli, T. Charnikhova, H. Bouwmeester, C. Mariani, and W. H. Vriezen (submitted)

Abstract

ABA-deficient mutants, such as *notabilis* and *flacca*, have helped elucidating the role of ABA during stress responses in tomato (*Solanum lycopersicum* L.). However, these mutants are not totally ABA deficient. Here we report of the vegetative and generative development in *notabilis* and *flacca,* and in a *notabilis-flacca* (*not/flc)* double mutant. We found lower levels of ABA in the double mutant. Additionally, we observed that the response measured for plant growth, leaf-surface area, wilting and ABA related gene expression in the different ABA-deficient lines was in accordance with ABA levels. Flower bud growth was similar in ABA-deficient and wild-type lines, but fruit set was reduced in the *not/flc* double mutant lines. However, when flowers were hand pollinated with wild-type pollen no significant differences in fruit set could be observed between any of the lines. Despite, we could not find differences in pollen germination, *in vitro* or *in vivo* or in anther and pollen morphology between wild-type and ABA-deficient lines. Fruit size was smaller in the ABA-deficient lines, most likely due to the reduced cell size in *not/flc* double mutants.

Introduction

Abscisic acid (ABA) is well known as a hormone involved in stress responses. It is involved in the response to many abiotic stresses most notably drought stress and salt stress, but also in the response to biotic stresses (Wasilewska *et al.* 2008; Mauch-mani and Mauch, 2005; Seki *et al.* 2007). Depending on the type of stress, ABA can induce the expression of stress-related genes, induce metabolic changes and have a direct effect on stomatal closure to reduce water loss (Seki *et al*. 2007). ABA deficient mutants have played an important role in elucidating these functions (Schwarz *et al.* 2003).

In tomato there are three ABA-deficient mutant lines available; s*itiens, flacca*, and *notabilis.* For *flacca* and *notabilis* the mutated genes and the corresponding mutations have been elucidated. The *notabilis* mutant contains a frameshift mutation in the *LeNCED1* gene (Burbidge *et al*. 1999) leading to a null mutation and decreased 9-*cis* epoxycarotenoid dioxygenase (NCED) activity (Thompson *et al*. 2004). NCED catalyzes the first committed step of ABA biosynthesis (Qin and Zeevaart 1999; Taylor *et al.* 2000). The mutation in the *flacca* mutant is a deletion of 6 base pairs in a Molybdenum-cofactor sulfurase (Sagi *et al*. 2002). This mutation reduces the oxidizing capacity of both aldehyde oxidases (AOs) and xanthine dehydrogenases (XDHs). AOs catalyze the final step in ABA biosynthesis, the conversion of abscisic aldehyde to ABA. Although both mutants are markedly impaired in

their ability to synthesize ABA, they still contain considerable levels of ABA, respectively 47% in *notabilis* and 21% in *flacca* as compared to the levels in wild type (Herde *et al.* 1999).

Xiong and Zhu (2003) mentioned that because ABA-deficient mutants contain ABA levels that are not dramatically lower than those in wild type under normal growth conditions, it is difficult to uncover cellular processes that require a very small amount of ABA. A double mutant line might therefore help to identify such processes, because it is expected to have even lower levels of ABA than the known ABA deficient mutants. Taylor and Tarr (1984) produced several double mutant lines of *notabilis, flacca* and *sitiens*. They stated that these double mutant lines may provide invaluable information on the physiological role of ABA in plants (Taylor and Tarr 1984). However, there are no records of the use or of further phenotypic description of these double mutants. Therefore, we set out to study the effect of very low ABA levels on growth and developmental processes by making a new *notabilis-flacca* (*not/flc*) double mutant.

Although several studies have been made on the ABA-deficient single mutant lines of tomato the focus has mainly been on stress responses, while it is known that ABA is also involved in many developmental processes such as seed maturation, seed dormancy and bud dormancy (Finch-Savage and Leubner-Metzger 2006; Ruttink *et al.* 2006; Wasilewska *et al.* 2008). To that list of developmental processes recently a possible function for ABA in the mature ovary and/or in early fruit development could be added. We found ABA-related genes to be highly expressed in mature ovaries as were ABA levels and ABA biosynthesis (Vriezen *et al.* 2008; Chapter two). Altogether these data suggest a function for ABA in flower development and especially in the ovary shortly before and during full bloom. To get a better insight in the role of ABA in flower and fruit development we analyzed several aspects of these processes in the tomato ABA deficient mutants; *notabilis*, *flacca* and *not/flc* double mutants.

Results

Isolation of the *not/flc* double mutant

The two mutations of the *notabilis* and *flacca* mutants have both been localized on chromosome 7 (Taylor and Tarr 1984). The primary cross was made between a homozygote *notabilis* and a heterozygous *flacca* plant. We selfed the progeny of this cross and the F2 was screened by two PCR-based methods. To detect the *notabilis*-mutation, primers were designed to amplify a 390 base pair product that spans this mutation. The PCR fragment from wild type DNA contains the recognition site of *TSp*RI endonuclease (New England Biolabs) and can therefore be digested in contrast to the PCR product of the mutant allele that contains a disrupted recognition site. The *flacca*-mutation was detected by separating a PCR fragment obtained with primers that span the *flacca*-mutation on a polyacrylamide-gel. Four different *not/flc* double mutant lines were isolated that were homozygous for both mutations. At least two of these lines were used for all the experiments. If measurements were averaged all four lines of the *not/flc* double mutants were used.

ABA levels and ABA response gene-expression in *not/flc* double mutant

notabilis and *flacca* are impaired in different steps of the ABA biosynthesis route, therefore the double mutant is expected to have lower ABA levels than either one of the respective wild type or single mutants. We measured the ABA levels in unstressed leaves and we used leaf detachment as a way to stress leaf tissue. ABA levels in non-stressed leaves of all four double mutant lines were lower as compared to both wild-type and single mutant plants. Likewise, leaves of *notabilis* and *flacca* contained lower levels of ABA than their wild types, Ailsa Craig (AC) in case of *notabilis* and Rheinlands Ruhm (RR) in the case of *flacca* (Fig. 1A). ABA levels were significantly higher in the stressed leaves as compared to the non-stressed leaves in the two wild types but not in the ABA-deficient lines (Student's T-test, $p<0.1$). In mature unpollinated ovaries the ABA levels were significantly (Student's T-test, $p<0.05$) different between AC and RR (Fig. 1B). The ABA level in the ovaries of *notabilis* and wild type AC were not different, but the ABA levels in the ovaries of wild type RR were higher than those in the ovaries of *flacca*. The *not/flc* double mutant plants had the lowest ABA level in ovaries. Interestingly, the ABA levels in unpollinated ovaries of AC and RR (0.72 and 1.1 ng/mg FW) were comparable to the ABA levels of stressed leaves of AC and RR (0.97 and 0.94 ng/mg FW).

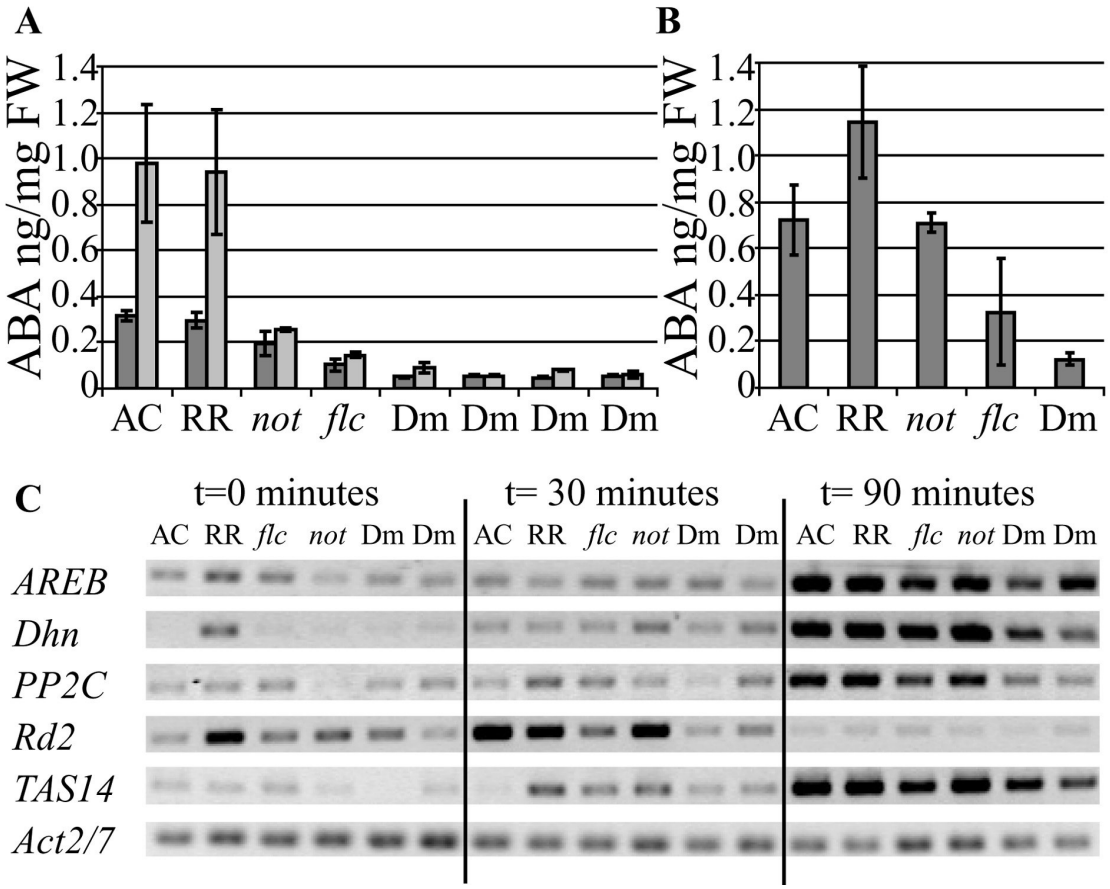


Fig. 1: A) ABA concentration in leaves (dark grey bars) and in stressed leaves (light grey bars). Wild type leaves (AC and RR) have a higher level of ABA than single mutants (*not* and *flc*) and all four *not/flc* double mutant lines (Dm) in non-stress and stress conditions. Wild-type leaves also show a stronger increase in ABA levels after detachment (stress) than single or double mutants do. **B)** ABA concentration in wild-type unpollinated mature ovaries are comparable to ABA levels in stressed leaves. Wild types have higher ABA levels than *flc* and *not/flc* double mutants. The data of the four *not/flc* double mutant lines are combined. **C)** Gene expression of ABA-responsive genes after 0, 30 and 90 minutes of detachment (stress). All genes are higher expressed after stress. However, the increase in gene expression is lower in single and double mutants. Mean ABA concentrations with SE are shown. A representative ethydium-bromide stained gel is shown for gene expression.

Although the ABA concentration was significantly reduced in all four double mutant lines, its effect on ABA responses could in theory be partly annihilated by changes in ABA sensitivity. We therefore also tested five ABA-response genes to confirm that not only the concentration of ABA was reduced in the *not/flc* double mutant, but that consequently ABA-response genes were also expressed at lower levels. Figure 1C shows that mRNA levels of response genes in leaves were not clearly different between wild types, single and double mutants under control conditions (t = 0). However, 30 or 90 minutes from detachment a

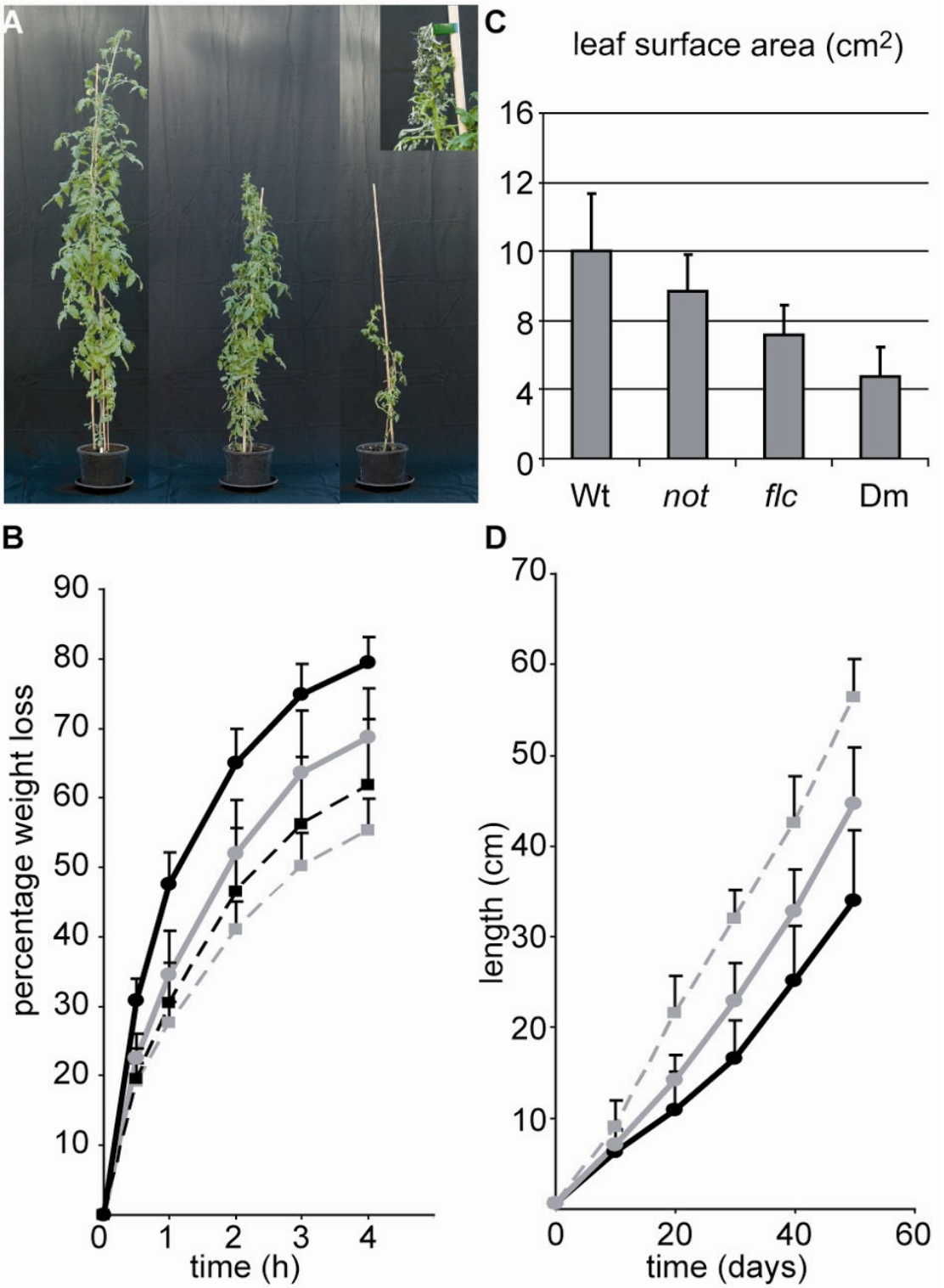
strong increase of the mRNA levels of all ABA- responsive genes was observed. The effect was generally most pronounced after 90 minutes. Only the mRNA levels of the gene *Rd2* (*Rapid dehydrin 2*) reached a maximum after 30 minutes and decreased after that. In the two *not/flc* double mutant lines the increase in the mRNA levels of all five genes tested was less pronounced as compared to *flacca* and *notabilis,* which had in turn a less pronounced increase in mRNA levels than wild-type plants.

Biometric analysis

It has been shown before that the *notabilis* and *flacca* mutant plants are smaller than their wild types (Taylor and Tarr 1984). Figure 2A shows five months old wild-type (RR), *flacca* and *not/flc* double mutant plants (left to right). The double mutants are smaller than *flacca* and the wild types, and have s stronger wilty phenotype (inset). Figure 2B shows the weight loss of detached leaves, most likely corresponding to water loss. *flacca* and *not/flc* double mutants lost significantly (Student's T-test, $p<0.05$) more weight than wild type and *notabilis* showed a similar trend. To minimize the influence of wilting during growth and during the experiments, we grew plants under high relative humidity (~ 70%). However, even under these conditions, differences in growth rate were observed. Figure 2C shows that the mean leaf surface area was reduced in both *flacca* and *not/flc* double mutants, no significant differences were obtained for *notabilis* as compared to wild type. Also plant length was reduced under high relative humidity (Fig 2D). After 20 days or more, the wild-type plants were significantly (Student's T-test, $p< 0.05$) taller than both *flacca* and *not/flc* double mutants. The length of the *notabilis* plants was comparable to wild type (data not shown).

Next page:

Fig. 2: A) Rheinlands Rhum, is taller than *flacca* and *not/flc* double mutant line (left to right), severe wilting in the double mutant apex (inset). **B)** Weight loss after leaf detachment is increased in the single and double mutants, wild type (grey dashed line), *notabilis* (black dashed line), *flacca* (grey solid line) and not/flc double mutants (black solid line). **C)** Mean leaf surface area is reduced in *notabilis* (*not*), *flacca* (*flc*) and *not/flc* double mutants (Dm). **D)** Wild-type plants are taller than ABA-deficient mutants. Lines and symbols similar to (B), mean values of biological repeat and SD are shown.


A
C
leaf surface area (cm2)
16
12
10
8
4
0
Wt
not
flc
Dm
B
percentage weight loss
90
80
70
60
50
40
30
20
10
0
1
2
3
4
time (h)
D
length (cm)
70
60
50
40
30
20
10
0
20
40
60
time (days)

Fruit set

Under ambient-greenhouse conditions (relative humidity ~50 %) the percentage of *not/flc* double mutant flower buds that formed fruits was significantly lower (Student's T-test, $p<0.05$) than the percentages in both wild types (Fig. 3A). Under these conditions, premature wilting of the flower buds reduced the number of open flowers on double mutant plants. However, when wilting was prevented by growing plants under high relative humidity (~ 70%), flower development was similar to wild type. We measured the percentages of fully opened flowers that formed fruits under high relative humidity. Figure 3B shows that the percentages of fruit set on the different lines had a comparable pattern as under ambient greenhouse conditions. However, the percentage of flowers that set fruit on the *not/flc* double mutants and *flacca* was low, but not significantly different from wild type RR. We also measured fruit set after hand pollination with wild type (Moneymaker) pollen, from plants grown under ambient greenhouse conditions. No significant differences in fruit set percentages between either of the wild types and ABA-deficient lines could be observed (Fig. 3C). This suggests that the low fruit set percentages resulted from bad pollen quality.

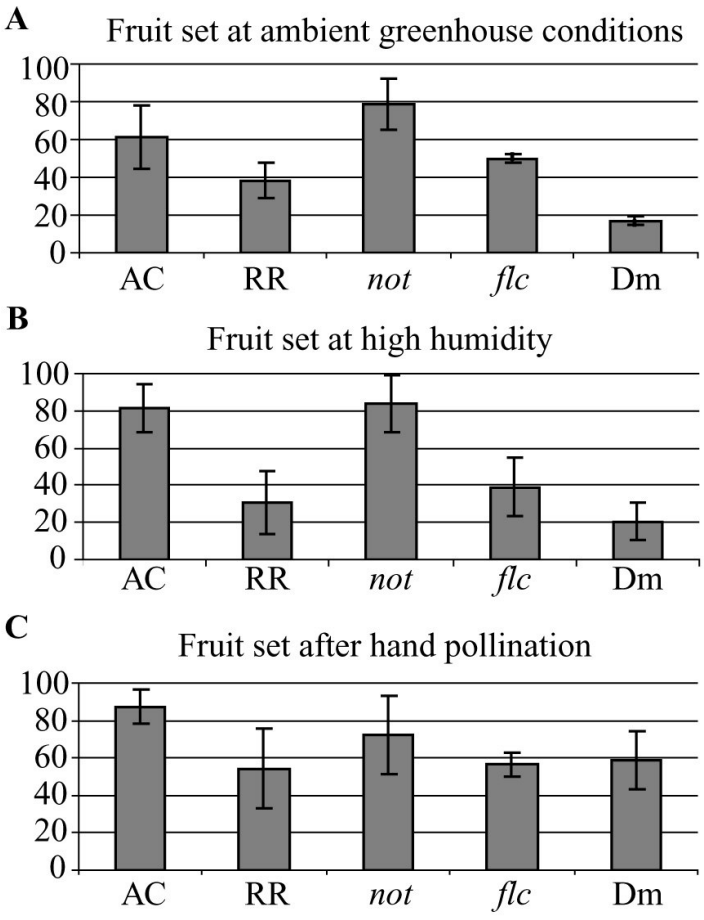


Fig. 3: A) Fruit set at ambient green house conditions (~50%) is significantly reduced in the *not/flc* double mutant lines (Dm) compared to Ailsa Craig (AC) and Rheinlands Ruhm (RR). **B)** Under higher relative humidity (~70%) fruit set is low in RR, *flacca* (*flc*) and *not/flc* double mutant lines. **C)** After hand pollination with wild type (Moneymaker) pollen no significant differences could be found. *notabilis* (*not*) and *flacca* are never significantly different from their respective wild type.

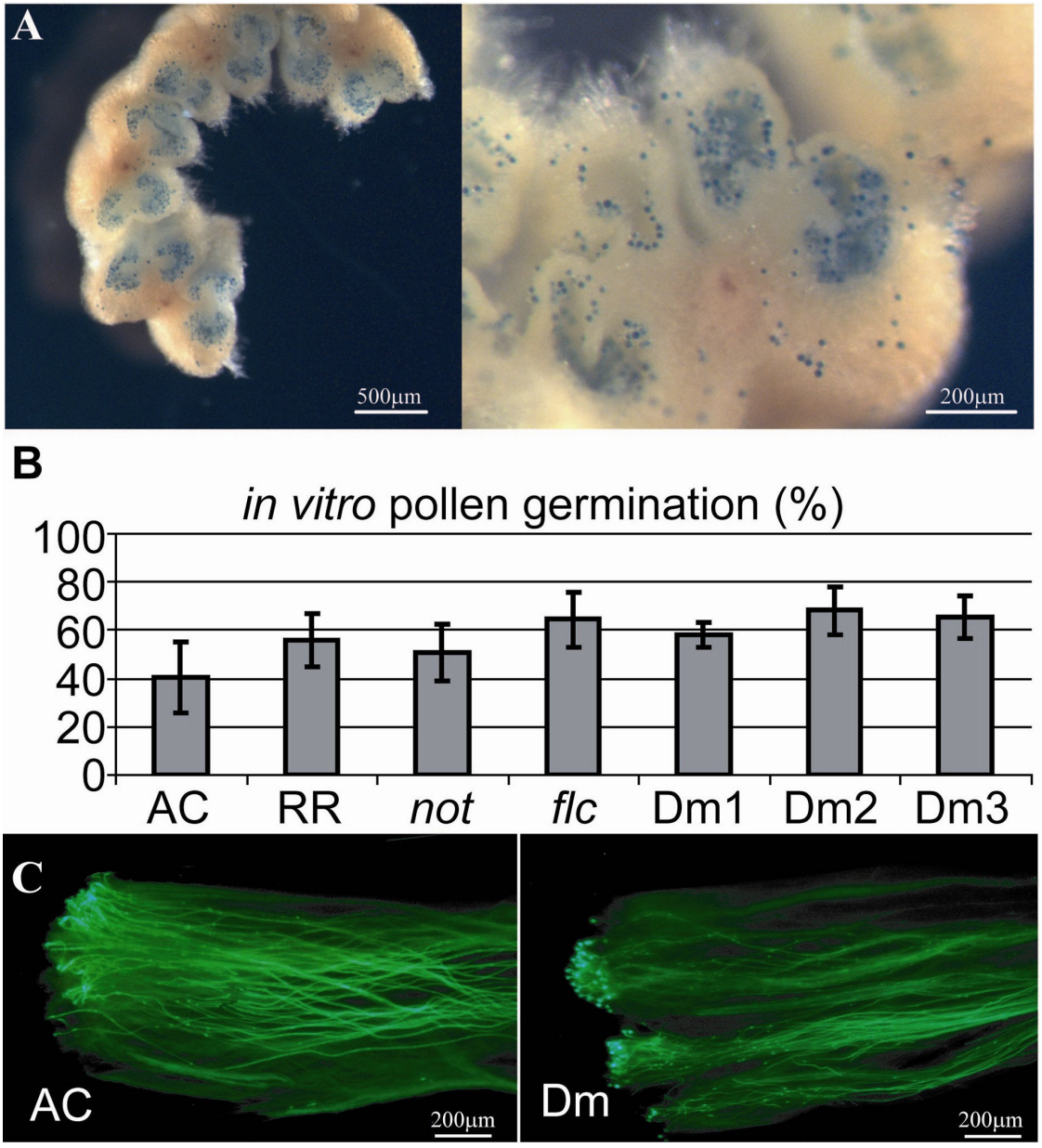


Fig. 4: A) GUS staining (blue) is observed in ~50% of the mature pollen of this heterozygous transgenic line, harboring a construct with the *uidA* gene (GUS) under control of an ABA-responsive promoter (*RD29B*). No other anther tissues are stained. **B)** *in vitro* pollen germination is similar in RR and ABA-deficient lines. AC has lower pollen germination rates. **C)** *in vivo* pollen germination was observed in a wild type (Moneymaker) style with pollen from both wild type (RR and AC) and double mutant plants.

To determine whether ABA signaling might play a role in pollen development, we analyzed the expression of the Arabidopsis ABA responsive promoter *RD29B* driving the *uidA* gene (GUS) in tomato. Strong GUS staining was observed in the pollen of mature anthers (Fig. 4A). Half of the pollen in this heterozygous line were blue, no staining was

observed in the other anther tissues. FESEM of the anthers of wild type and ABA-deficient tomato lines revealed that mature dehydrated pollen were present in the anthers of all lines. No aberrant anther morphology was observed and anther dehiscence occurred normally. We also measured the pollen germination capacity *in vitro* and found that there was no reduction in germination percentages in *flacca* or the *not/flc* double mutants as compared to wild type RR (Fig. 4B), though pollen germination increased in the *not/flc* double mutants as compared to AC. In *vivo* pollen tube growth of pollen from both wild type and *not/flc* double mutant lines in the style of wild type (Moneymaker) flowers was examined (Fig. 4C). No significant differences were observed between any of the lines.

Fruit development

Fruit weight was strongly reduced in double and also in the single mutant lines (student's T-test, $p<0.005$, $n>30$, Fig. 5A). The size reduction was observed when fruits were grown on the plant either in ambient greenhouse conditions or at high relative humidity. We measured the diameter of the fruits during fruit growth and found that the double mutant lines have significantly (ANOVA, $p<0.05$) reduced fruit diameters throughout development compared to wild type. However, the differences are most obvious at later stages of fruit development (Fig. 5B). The size reduction was observed in all tissues of mature fruits; placenta, locule, and pericarp (Fig. 5C). The pericarp width was significantly (student's T-test, $p<0.005$) reduced in the double mutant lines, which had on average 4.0 mm thick pericarp, as compared to 5.3 mm in AC and RR (wild type). To determine if the difference in size occurred mainly due to reduced cell division or cell enlargement, we quantified cell size and the number of cell layers in the pericarp of tomato fruits at breaker stage. No difference in the number of cell layers in the pericarp was found; all lines had 14 to 15 cell layers. However, cell size was significantly (student's T-test, $p<0.05$) reduced in the pericarp of the *not/flc* double mutant lines as compared to wild type (Fig. 5D,E). Cells of wild type RR and AC had a mean area of $33\mu m^2$ and $38\mu m^2$, respectively, as compared to $20\mu m^2$ and $25\mu m^2$ in the two double mutant lines. The cells were divided in ten size categories, the percentage of cells that fell into a certain category and the size-limits (μm^2) of the different categories are depicted in figure 5F. A larger percentage of the pericarp cells of double mutant fruits falls into the categories with the smallest cells (category 1 and 2) as compared to wild type fruits, whereas a considerable amount of cells of wild-type fruits falls into the size categories with the larger cells (category 5-10).

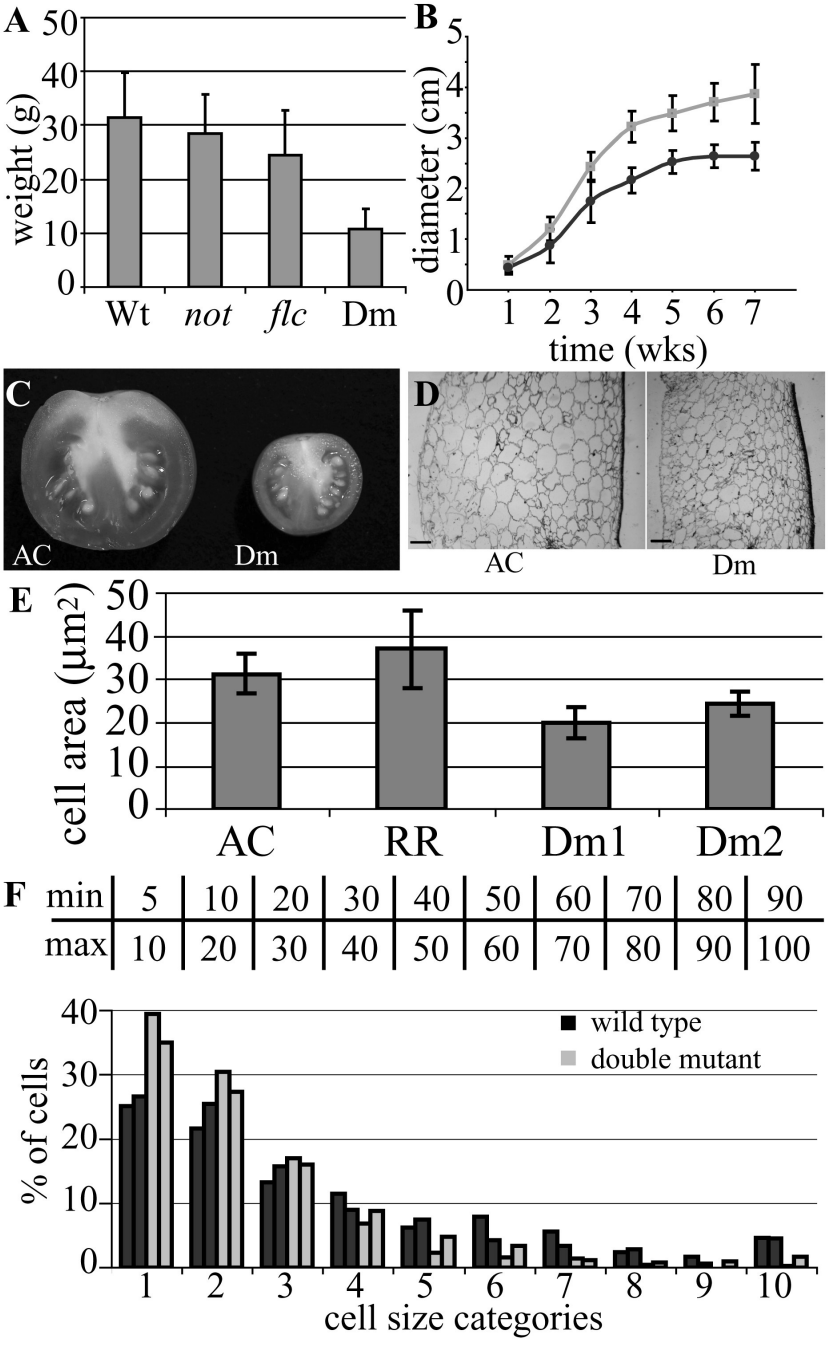


Fig. 5: A) Mean weight (g) of the fruits of *notabilis* (*not*), *flacca* (*flc*) and *not/flc* double mutant lines (Dm) is significantly (Student's T-test, $p<0.005$) smaller than wild type (Wt). **B)** Increase in diameter of tomato fruits of wild type and *not/flc* double mutant lines was measured during fruit development. Wild type fruit size (grey) is bigger than fruit size of *not/flc* double mutants (black). **C)** Mature fruits of Ailsa Craig (AC, left) and a *not/flc* double mutant line (Dm, right) show that the pericarp, locule en placenta sizes are smaller in Dm. **D)** Aniline blue stained pericarp of Ailsa Craig (right) and a *not/flc* double mutant (left). Pericarp cells are smaller in the *not/flc* double mutant line. Bar represents 200µm. **E)** Pericarp cell size is smaller in the *not/flc* double mutant lines (Dm) than in both wild types (AC, RR). **F)** Percentage of cells grouped into ten size categories in wild type lines (black bars) and *not/flc* double mutant lines (light grey bars). Minimal and maximal cell size (μm^2) in the different categories is noted in the table above the graph. The percentages of cells in the categories with the smallest cells (1-2) is higher in the *not/flc* double mutant than in wild type. In the categories with the larger cells (7-10) a higher percentage of wild-type cells is found.

Discussion

ABA levels and biometric analysis

The differences in ABA levels that we measured under non-stressed conditions in leaves of *notabilis, flacca* and wild type are comparable to those measured by Herde *et al.* (1999) and Tal and Nevo (1973). The *not/flc* double mutants had lower ABA levels than the single mutants both in leaves and ovaries. Remarkably, the ABA level in wild type unpollinated mature ovaries is relatively high and comparable to a stress situation in leaf, perhaps indicating that unpollinated mature ovaries are in a stress-like situation. The differences in ABA levels between the different ABA-deficient lines (*notabilis, flacca* and *not/flc* double mutant) correlate with the differences measured for plant length, leaf surface and water loss. The ABA-deficient plants with the highest amount of ABA, *notabilis*, differed slightly in phenotype, while *flacca* differed clearly in phenotype from both wild-type lines. The double mutants, which have the lowest ABA level and lowest expression of ABA-response genes, are also most impaired in growth and water balance. This probably indicates that all three characteristics are affected by ABA in a dose dependent manner, and not by threshold levels. Interestingly, it has long been thought that ABA inhibited shoot growth, but it was recently shown that ABA-deficient *notabilis* or *flacca* when grown under controlled humidity conditions had markedly impaired shoot and root growth parameters (Sharp *et al*. 2000). We obtained similar results in our experiments. The double mutants remained impaired in their growth and leaf surface area under high relative humidity. The growth inhibition of *flacca* and *notabilis* as compared to wild type was caused by an increased ethylene evolution rate (Sharp *et al.* 2000, 2002). Possibly this is also the reason for the growth reduction in the *not/flc* double mutants, but this remains to be tested.

Fruit set and development

The reduction in fruit set percentage of *not/flc* flowers under ambient-greenhouse conditions, might be explained by wilting and abscission of the flowers of these plants under these conditions. However, when plants were grown at relative higher humidity (~70%) fruit set did not significantly increase in the *not/flc* double mutants. Therefore, it seems that water stress was not a major factor decreasing fruit set in these mutants. It remains however very difficult to draw conclusions about the influence of reduced ABA levels on fruit set. After all, the fruit set of the wild type RR was also very low and significantly different form the other wild type AC under the conditions we tested. The low level of fruit set in RR remains

puzzling, since RR is described as a cultivar that carries many fruits. After hand pollination with wild type pollen no significant differences in fruit set percentages between the different lines were observed, suggesting that the reduced fruit set was caused by less viable pollen. Accordingly, GUS staining in the pollen of transgenic plants expressing *RD29B-GUS*, suggests a strong ABA signal at the end of pollen development. Moreover, Peng *et al.* (2006) detected ABA by immunolocalization in the nursing cells of anthers and ovules, tapetum and integuments, during flower development in *Arabidopsis* and cucumber, and stated that ABA has a positive role in pollen development. ABA deficiency may thus have a negative effect on pollen development. However, we did not find any significant differences in pollen germination rates *in vitro* or *in vivo*, or in anther and pollen morphology between wild type and ABA-deficient lines. Most likely, the defects in pollen development are thus only apparent during or after fertilization, or the effects of ABA deficiency are small and therefore not detected by us.

A function for ABA in the unpollinated ovaries did not become apparent from our research on the ABA biosynthesis mutants. However, the ABA levels in wild-type ovaries are comparable to those in stressed leaves. In addition, the ABA levels in the ovary are strongly regulated before and after pollination (chapter two). Moreover, our previous results showed that also ABA responsive genes such as genes involved in protection against drought are active in the ovary (Vriezen *et al.* 2008). Altogether, it seems likely that the function of ABA is conditional and only apparent under specific environmental conditions, not tested by us.

We observed smaller fruits in all ABA-deficient lines. The fruit size reduction was stronger in the *not/flc* mutants than in the single mutants, indicating a concentration-dependent effect of ABA. The reduction in fruit size may be explained by either reduced cell division or reduced cell enlargement. It is known that ABA can directly influence the number of cell divisions via its influence on cell cycle genes such as *CDKA1* (Smalle *et al.* 2003) or *ICK1* (an inhibitor of CDK action) (Wang *et al.*1998). Additionally, Zhang (2007) concluded that higher ABA levels in Japanese pear were correlated with low cell division. Both observations would imply that ABA-deficient plants would have larger fruits, opposite to our observations. We could also not detect any significant differences in the final number of cell layers in the pericarp at breaker stage between the ABA-deficient and wild type plants. The mean cell area we measured is in accordance with a range of diameters measured for twenty tomato species by Cheniclet *et al.* (2005), ranging from 10 to 350 µm. The mean size of the pericarp cells in the *not/flc* double mutants was significantly smaller than the mean cell size in both wild types, indicating that ABA probably promotes cell enlargement. Consistently, ABA

levels increase at the beginning of the cell expansion phase and peak around the middle of the cell expansion phase (Sjut and Bangerth 1982/83). In the past it has been observed that ABA has a positive effect on dry matter deposition during seed and grain filling (Yang *et al*. 2006a, b). Additionally, Kojima (2005) stated that ABA may stimulate phloem unloading in the placenta and promote sink activity in the pericarp and locule tissue during the high growth rate phase. ABA thus might stimulate fruit growth through stimulation of cell enlargement by increasing sink strength and dry matter deposition. The size reduction of the fruits of the ABA-deficient lines might also be attributable to the smaller amount of seeds that were present in these tomatoes, since it is known that final tomato size is correlated with the number of seeds (Gillaspy *et al.* 1993; Nitsch 1970). However, smaller fruits were also observed in the double mutant lines after fruit induction by gibberellins. Moreover the number of seeds per gram of fruit tissue is higher in the mutants (on average four) than in the wild type (on average two). Together this leads us to the conclusion that the reduction of seed number might have contributed to the fruit size reduction in the double mutants but is not the only cause. We also concluded that ABA seems to be stimulating fruit growth by stimulating cell enlargement. Auxins have also been implicated in cell enlargement and they are thought to cause an increase in the extensibility of cell walls and induce uptake and retention of water and solutes (Hackett and Thimann 1952). However, in parthenocarpic fruits decreased cell enlargement could not be counteracted by auxin application (Gillaspy *et al.* 1993), it was therefore proposed that in addition to auxin another regulatory molecule may be produced, which may direct the sink activity of cells in the pericarp, placenta, and locular tissues. The concerted action of both molecules could then give rise to the increase in cell volume (Gillaspy *et al.* 1993). The results discussed above suggest that this molecule is ABA.

Concluding remarks

The *not/flc* double mutant lines indeed produced less ABA than either of the wild-type or single mutant lines. They have been proven to be extremely convenient in detecting dose-response effects of ABA on leaf area, plant growth and water loss. They also enabled us to detect a function for ABA in fruit growth. This was more difficult to detect in single mutant lines, and large numbers of fruits had to be measured before we could observe significant differences as compared to wild type. Altogether the *not/flc* double mutant seems to be a powerful tool to uncover the cellular processes that require a small amount of ABA and they might reveal the involvement of ABA in many more developmental processes.

Acknowledgement

We would like to thank Dr. A. Christmann for supplying us with the *RD29B-GUS*-constructs. We also would like to thank Dr. R. de Maagd and Mr. M. Busscher for their assistance with the cell size analysis.

Materials and Methods

Plant material

Moneymaker seeds were obtained from Enza zaden (Enkhuizen, the Netherlands) while seeds of Ailsa Craig, Rheinlands Ruhm, *notabilis* (accession number LA3614, genetic background Ailsa Craig) and *flacca* (accession number LA0673, genetic background Rheinlands Ruhm) were obtained from the Tomato Genetics Resource Centre (TGRC, University of California, Davis, USA). Seeds were surface sterilized in 2% Hypochlorite with 0.1% tween and washed in distilled water before sowing on MS medium containing Nitsch vitamin mixture (Duchefa, Haarlem, the Netherlands), 1.5% sucrose and 0.7% agar. Seeds were germinated at 25 ^{o}C and transferred to the green house after two weeks, where they were grown at a humidity of 95-98% until they were four to five weeks old. Most plants were afterwards transferred to an isolated cabinet in which humidity was on average 70% (varying between 60%-80%) and grown further under 16/8h day-night rhythm. Supplementary lights (600 Watt high pressure sodium lights) turned on below 200 W/m^2 and turned off above 300 W/m^2. Temperature was kept above 20°C during the light period and 17°C during the dark period with the PRIVA Integro versie 724 system. Plants were watered daily and given fertilizer weekly. Plants that were grown under ambient-greenhouse conditions were sown as described above but when they were four to five weeks old, they were transferred to the normal green house were relative humidity was on average 50% (between 30%-70%). All the other conditions were similar to the conditions under higher relative humidity, described above.

Isolation of the double mutant

A homozygous line for *notabilis* was obtained by selfing the heterozygous plant and screening the progeny with a PCR-based method. gDNA of the plants was isolated as described by Edwards *et al.* (1991), cleaned by phenol-extraction and dissolved in water. A

PCR was performed with primers (5'- GCAGAAAGCAGCA GCAATGG-3' and 5'-CCAGAGTGACCATGTAATTCA-3') designed to span the *notabilis* mutation. Five µL gDNA, buffer IV, 2.5 m*M* $MgCl_2$, 0.5unit redhot Taq DNA polymerase (all from ABgene limited, Epsors, UK), 0.4 m*M* dNTPs (Fermentas, St Leon-Rot, Germany), and 0.1 µ*M* primers were used in a reaction of 30 cycles, each comprising of 30 seconds at 95°C (denaturation), 30 seconds at 58°C (primer annealing) and 90 seconds at 72°C (extension time). The PCR products were digested with 1U TSpRI (R0582) in NEB4-buffer with 0.1 mg/mL BSA (all from New England Biolabs, Ipswich, MA, USA) at 65 °C for eleven hours. Fragments were visualized on an agarose gel containing ethidium-bromide (25µg/L).

A primary cross was made between a homozygote *notabilis* and a heterozygous *flacca* plants. The progeny of this cross was selfed and the F2 was screened for the *notabilis* mutation (as described above). The *flacca*-mutation was detected by a PCR with primers (5'-CAAGTCTCAAATAGGAACTTCCAC-3' and 5'- TTCGACCCAATCTTG TAGCA-3') designed to span the *flacca*-mutation. gDNA was isolated, cleaned and a PCR was performed as described above, but with 40 cycles, each comprising of 30 seconds at 95°C, 30 seconds at 55°C and 30 seconds at 72°C. PCR fragments were separated on a 10% polyacrylamide gel (Bio-Rad laboratories, Hercules, CA, USA).

ABA extraction

All samples were harvested in June between 11.00-13.00 hours and directly frozen in liquid nitrogen. For measurements under stress conditions, leaves were detached from the plants and left on the laboratory bench for 90 minutes before sampling. Leaves tissue (100-300mg) or ovary tissue (30-60mg) were ground to powder in liquid nitrogen. Before the extraction [$^2H_6$]*(+)-cis,trans*-abscisic acid was added to the samples as the internal standard (0.1nmol to the leaf or 1nmol to the ovary tissue, respectively). The samples were extracted twice with 1ml of cold ethyl acetate. Organic fraction were combined, and dried under nitrogen stream. The residue was dissolved in 100µL (ovaries) or 200µL (leaves) of acetonitrile: water:formic acid (25:75:0.1). The samples were filtered through Minisart SRP4 0.45µm filters (Sartorius, Germany) and the aliquots of them were used for LC-MS/MS analysis.

ABA detection and quantification by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

ABA detection and quantification was performed by LC-MS/MS as described previously (Saika *et al.* 2007; Filaro *et al.* 2006) with some modifications. Analyzes were conducted using a Waters Micromass Quattro Premier XE tandem mass spectrometer (Waters, Milford, MA, USA) equipped with an ESI source and coupled to an Acquity UPLC system (Waters, USA). Chromatographic separation was achieved using an Acquity UPLC BEH C_{18} column (150 x 2.1 mm, 1.7 µm) (Waters, USA), applying a water/acetonitrile gradient, starting at 0% acetonitrile for 2.0 min, raised to 50% acetonitrile in 8.0 min, followed by a 1.0 min gradient to 90% acetonitrile which was then maintained for 0.1 min and followed by a 0.2 min gradient back to 0% acetonitrile before the next run. The column was then equilibrated at this solvent composition for 2.8 min. Total run time was 15 min. The column was operated at 50ºC with a flow-rate of 0.4 ml min^{-1} and sample injection volume was 30 µL. The mass spectrometer was operated in positive and negative electrospray ionization (ESI) mode. The nebuliser and desolvation gas flows were 50 and 800 L h^{-1}, respectively. The capillary voltage was set at 2.7 kV, the cone voltage at 10 V in positive and 30 V in the negative mode, respectively; the source temperature at 120ºC and the desolvation gas temperature at 450ºC. Fragmentation was performed by collision induced dissociation with argon at $3.0x10^{-3}$ mbar. Multiple reaction monitoring (MRM) was used for ABA quantification and the MRM transitions were set according to the MS/MS spectra obtained for the standards ABA and [$^2H_6$]-ABA. The compounds gave better response in the positive than in the negative ESI mode, therefore MRM parent-daughter transitions in positive mode were selected. Protonated molecular ions $[M + H]^+$ were selected as parent ion for the transitions. Two parent-daughter transitions were selected for each ABA and [$^2H_6$]-ABA, according to the most abundant and specific fragment ions for which the collision energy (CE) was optimized. For ABA, the MRM transitions *m/z* 265>229 at a CE of 10 eV and 265>247 at 5 eV; and for [$^2H_6$]-ABA, the transitions *m/z* 271>234 at 10 eV and 271>253 at 5 eV were selected.

The ABA was quantified using a calibration curve with known amount of standards and based on the ratio of the summed area of the MRM transitions for ABA to those for [$^2H_6$]-ABA. Data acquisition and analysis were performed using MassLynx 4.1 software (Waters, USA). The summed area of all the corresponding MRM transitions was used for statistical analysis.

Semi-quantitative RT-PCR of ABA responsive genes

ABA-induced genes were selected from literature (Uno *et al*. 2000; Del Mar Parra *et al*. 1996; Vriezen *et al.* 2008) and specific primers (Table 1) were designed using a computer program (Beacon Designer Software, Premier Biosoft International, CA, USA). Leaves were harvested between 11.00-13.00 hrs. Several leaves were detached from the plants and left on the laboratory bench for 30 or 90 minutes before sampling to induce stress. RNA was isolated with Trizol reagent^{tm} (Invitrogen, Carlsbad, CA, USA). Photometric RNA measurements were done to equilibrate the RNA concentrations of different samples. Equal amounts of RNA were DNase treated with RNAse free DNase (RQ1, Promega, Madison, USA). RNA (0.5 µg) was reverse transcribed (RT) using a cDNA synthesis kit (iScript^{tm}, Bio-rad Laboratories, Hercules, CA, USA) following protocol. Semi-quantitative-PCRs were performed using 5 µL of 25-fold diluted cDNA, buffer IV, 2.5 m*M* $MgCl_2$, 0.5unit redhot Taq DNA polymerase (all from ABgene limited, Epsors, UK), 0.4 m*M* dNTPs (Fermentas, St Leon-Rot, Germany), and 0.1 µ*M* primers in a reaction of 24-30 cycles, each comprising of 15 seconds at 95^{o}C (denaturation), 30 seconds at 57^{o}C (primer annealing) and 10 seconds at 72^{o}C (extension time).

Table 1 primer sequences of ABA-responsive genes

Name (genbank)	Forward primer	Reverse primer
AREB (AY530758.1)	5'-GCACTCAACTCTA ATTTCATTCAAGG-3'	5'-GTACTGTATTTCCTG CCTCTTAAACC-3'
Dhn (BG630475.1)	5'-CCATATATCAAACT TCATACACATAAGG-3'	5'-AAATTAAGGAGAAA TTACCAGGATACC-3'
PP2C	5'-TCGGAAGGAGAAG ATTACG-3'	5'-TCCACAATTCGCAA CAAC-3'
Rd2 (AI772677.1)	5'-TAATGCTGATTGG TATGATATAATGG-3'	5'-TTATAAACAAACTT AAATACTGAAGACC-3'
TAS14 (X51904.1)	5'-GGCACGGGTACTG GCGGCGGT-3'	5'-CCAGGCATCTTCTCC ATTATCTTCTCC-3'
Act2/7	5'-CATTGTGCTCAGT GGTGGTTC-3'	5'-TCTGCTGGAAGG TGCTAAGTG-3'

Detached leaf assay for water loss

Several leaflets of compound leaves were detached from the plant and weighted on t=0. The leaves were left on the laboratory bench (relative hum. 38%) and weighted every hour. Percentage weight loss compared to t=0 was calculated for every time point. At least eight leaflets were used per line. A student's T-test was performed to test for significant differences.

Plant growth rate assay and leaf-surface area

Main stalk of the seedlings and plants grown under high humidity (~70%) was measured every week for seven weeks. Mean of eight plants with SD is depicted. A student's T-test was performed to test for significant differences. Leaf surface area was measured for eight leaflets of 2 months old tomato plants on a Li-3100 area meter (LI-COR, Lincoln, USA). Mean leaf surface is depicted with SD. A student's T-test (p= 0.05) was performed to test for significant differences. Leaves were collected from the plants at comparable heights.

RD29B-GUS-transgenic lines and GUS-staining

We transformed tomato, as described by de Jong *et al*. (2008), with a construct (Christmann *et al*. 2005), containing the *uidA* gene (GUS) under control of an Arabidopsis *RD29B* promoter, which is ABA responsive. Transgenic plants were further selected based on tetraploidy test and by PCR with primers specific for the kanamycin resistance gene. Anthers of mature flowers were harvested and vacuum infiltrated with GUS-buffer containing 100 m*M* NaPO4 [pH 7.2], 0.5 m*M* potassium ferricyanide, 0.5 m*M* potassium ferrocyanide, 10 m*M* EDTA, 1% Triton X-100, and 2m*M* cyclohexyl-ammonium (XglcA, Duchefa, Haarlem, the Netherlands). Staining was performed overnight at 37˚C. The following day the tissue was cleared in choral-lacto-phenol (50% (w/v) chloral hydrate, 20% (w/v) lactic acid and 25% (w/v) phenol) and washed in lactic acid before mounting in lactic acid under the microscope.

Cryo-Field-Emission Scanning Electron Microscopy (FESEM)

For each line two transversal slices of anther-cones of two mature flowers were placed on a microscope disc in carbon paste and rapidly frozen in liquid-nitrogen. The frozen tissue was transferred to the vacuum chamber of the Cryo-field-Emission scanning electron

microscope (FESEM, JEOL-6330). After 4 minutes sublimation at -90˚C, the samples were sputtered with gold/platinum for 40 seconds at -140˚C, and examined in the microscope at -140˚C. FESEM was performed at the general instrumentarium of the science faculty (GA, Radboud University Nijmegen)

in vitro and *in vivo* pollen germination assay

In vitro pollen germination was tested as follows. An anther-cone of a mature flower was cut and 200µL pollen germination media (20m*M* Mes, pH 6,0, 3m*M* $Ca(NO_3)_2$, 1m*M* KCl, 0,8m*M* $MgSO_4$, 1.6m*M* boric acid, 2,5% (w/v) Sucrose and 24% (w/v) polyethylene glycol 4000) was added. Anther-cones were vortexed vigorously to release the pollen and incubated rotating for three hours at room temperature. Pollen-germination was examined under a light microscope at 10x objective. A minimum of 100 pollen grains was scored (normally in 10 frames). Six anther-cones per line were used. Mean germination percentages and SD are depicted.

Wild type (var. Moneymaker) flowers were emasculated three days before full bloom stage and pollinated with pollen from either, Ailsa Craig, Rheinlands Ruhm, or the *not/flc* double mutant lines, when the flowers had opened. Seven hours after pollination the styles were collected in a tube and 1*M* NaOH was added. Styles were incubated at 60^{o}C for two hours. The styles were washed in sterile water and squashed in a drop of aniline blue 0.1% (EMS, Hatfield, UK) in 2% $K_3PO_4*3H_2O$. Pollen-tube growth was examined under the fluorescent microscope.

Fruit set

(ambient greenhouse conditions) Number of fruits per number of flowers from ten inflorescences was measured. Fruit set was measured twice (in June and August), mean values and SD are depicted. *(high humidity)* Fruit set was measured as percentage of fruits formed from at least ten fully opened flowers per line, and was calculated one week after anthesis. Ovaries bigger than 3mm were scored as positive fruit set. Fruit set was measured twice (in June), mean values and SD are depicted. *(hand pollinated)* Ten flowers per line were emasculated and hand pollinated with pollen of wild type (variety Moneymaker) grown at ambient greenhouse conditions. One week after pollination fruits bigger than 3mm were scored as positive fruit set. Fruit set was measured twice (in august). A student's T-test was performed to test for significant differences.

Fruit size and fruit growth

Weight was measured from red tomato fruits which had developed under relative high humidity (~70%). Mean values of 20-50 fruits are depicted with SD. To measure fruit growth, we labelled ovaries between 3 and 5 mm that showed fruit induction and measured them once a week for seven weeks. Mean size of ten fruits of wild type or *not/flc* double mutant plants are depicted with SD. A repeated measure statistical test (ANOVA) was performed to test if the growth of the wild types differed from that of the *not/flc* double mutants ($p=0.031$).

Cell number and cell size

Pericarp tissues were collected from fruits that started to become orange (breaker stage) were bleached in 0.4% hypochlorite for 90 minutes and washed with sterile water. Pericarp tissue was fixated in FAA (3.7% formaldehyde, 5% acetic acid, and 50% ethanol) vacuum infiltrated for 15 minutes twice, and left overnight. Pericarp tissue was placed in 70% ethanol and stored until further processing. Fixed tissues were dehydrated with ethanol, cleared with xylene and embedded in parafin (Paraplast, Sigma-Aldrich, Steinheim, Germany). Embedded tissues were sliced into 8 µm sections. Paraplast was removed with xylene, sections were hydrated and stained in 4% toluidine blue staining (Acros, Belgium). Pictures were taken with 1.0 times objective. Contrast was manually adjusted in photoshop 7.0 to enhance cell wall recognition. Digital images of sections were analyzed using ImageJ (Rasband, 2008) in combination with two plug-in macros (available on request). With the first macro the image was pre-processed using the "skeletonize" operation to identify cell walls which are subsequently overlaid onto the original image. Subsequently missing cell wall parts were manually edited using ImageJ's drawing tools. A second analysis macro identified and selected cells by inversion of the edited overlay image and subsequently used ImageJ's "analyze particles" operation to measure the cell size parameters. Cell areas smaller than $5\mu m^2$ or larger than $100\mu m^2$ were excluded from the data, since they most likely represent intercellular spaces and cells that were disrupted. Mean cell size was calculated for each picture. Per line four pictures of different pericarp areas were averaged and depicted with SE.

Chapter 4

SlRPN10-silenced tomato plants have prolonged flower longevity and reduced ethylene emission rates

L. Nitsch, S. Cristescu, M. Wolters-Arts, C. Mariani and W.H. Vriezen

Abstract

Flower development in *SlRPN10*-silenced tomato plants seemed unaffected until the mature flower-stage. However, the unpollinated flowers of the transgenic tomato lines displayed delayed senescence as compared to unpollinated wild-type flowers. From two days before anthesis until five days after anthesis ethylene emission in transgenic flowers was lower which might be the cause of reduced senescence. Possible cross-talk between auxin, ABA and ethylene signaling during flower senescence is discussed.

Introduction

Fruit set is the commitment to continue with further fruit development and it is a highly regulated process. Normally it only occurs after successful pollination and fertilization, which are most likely the primary signals that promote fruit development (Gillaspy *et al.* 1993; Srivastava and Handa 2005). Pollination and fertilization can only occur if flowers have developed properly, and therefore flower development is very important for fruit development. More precisely, pollination and fertilization occur on the pistil, comprized of stigma, style and ovary, and the latter grows out into a fruit. The function of hormones during ovary development is not well understood. It is known that auxin is important for the patterning of the apical-basal axis of the ovary (Dinneny and Yanofsky 2005; Robles and Pelaz 2005) and that ethylene seems to play a role in ovule development in tobacco (De Martinis and Mariani 1999). In some species, most notably in orchids but also in maize, ovule development is induced by pollination and this is mediated by an increase in auxin and ethylene levels (Zhang and O'Neill 1993; Mól *et al.* 2004). Besides regulating ovule development in some species, in many species ethylene production after pollination has an additional role, it stimulates flower senescence (Shibuya *et al*. 2004; Van Doorn and Woltering 2008; Yang *et al*. 2008). A central role for the ovary in flower senescence has been established. It was shown that careful removal of the pistil resulted in increased flower longevity after pollination (Shibuya *et al.* 2000). Ethylene produced in the ovary after pollination is thought to induce a rapid increase in petal ethylene production, which ultimately induces senescence (Van Doorn and Woltering 2008). Flower senescence was proposed to guide pollinators to virgin flowers that still need to be pollinated (Van Doorn and Woltering 2008). Additionally, there are also indications that the whorls of anthers, petals and sepals might inhibit fruit set if they remain attached (Vivian-smith *et al.* 2001), indicating another possible explanation for senescence after pollination. Flowers senescence occurs also in the absence of pollination but this process is even less understood.

Abscisic acid (ABA) is a plant hormone, which is known to be involved in developmental processes such as apical bud dormancy and seed dormancy (Ruttink *et al.* 2007; Finch-Savage and Leubner-Metzger 2006). Additionally, ABA is well known for its function in abiotic stress responses (Bartels and Sunkar 2005). Moreover, recently we found that ABA-response and signaling genes were relatively highly expressed in the ovary of mature flowers of tomato, and less after pollination (Vriezen *et al*. 2008). Likewise, also ABA levels and ABA biosynthesis were high in the ovaries of mature flowers and decreased after pollination (chapter 2). This suggests that ABA is involved in processes during the later phases of ovary development or in fruit set. To gain more insight in the possible functions of ABA in flower and fruit development we decided to analyze these processes in ABA-hypersensitive plants.

In *Arabidopsis* the *rpn10-1* mutant was described to have an ABA-hypersensitive phenotype (Smalle *et al*. 2003). This mutant is affected in its 26S proteasome, which is responsible for protein breakdown. Subunit RPN10 is part of the 26S proteasome and is suggested to bind poly-ubiquinated proteins in a substrate-specific manner, and to stabilize the proteasome (Smalle *et al.* 2003). RPN10 exists also in an unbound form and might be involved in shuttling substrates to the proteasome (Smalle *et al.* 2003; Hartmann-Petersen and Gordon 2004). The ABA hypersensitivity phenotype of the *rpn10-1* mutant was observed in root elongation, and cotyledon expansion and cotyledon greening on ABA containing media. Other phenotypic characteristics known to be related to ABA such as reduced seed germination, reduced growth and lower fertility were described. Moreover, the ABA-signaling protein ABI5 was selectively stabilized in the *rpn10-1* mutant (Smalle *et al*. 2003). Although it was also shown that the Arabidopsis *rpn10-1* is slightly auxin and cytokinin insensitive, the authors concluded that this was likely to be caused by the ABA hypersensitivity (Smalle *et al.* 2003). We used an RNAi approach to silence the *RPN10*-homologue in tomato to obtain ABA hypersensitive transgenic tomato plants, in which we studied flower and fruit development.

Results

The *SlRPN10* RNAi knock-down plants

To obtain ABA hypersensitive tomato plants we silenced by RNAi a tomato gene (SGN-U216576, *SlRPN10*) whose deduced amino acid sequence shares 66% identity with AtRPN10. The 3`-end sequence, which contains the third ubiquitin interaction motive and the 3`-UTR region, was used to construct the RNAi-vector. Three transgenic lines, shown to be independent lines by southern blot analysis, were identified. They all had reduced *SlRPN10* mRNA levels in leaves (Fig. 1). Lines T5 and T44 also had a reduced mRNA level in unpollinated ovaries. Second generation plants, obtained from first line transgenic plants pollinated with wild type (Moneymaker) pollen, showed 1:1 (wt:transgenic) segregation of the kanamycin resistance marker (data not shown).

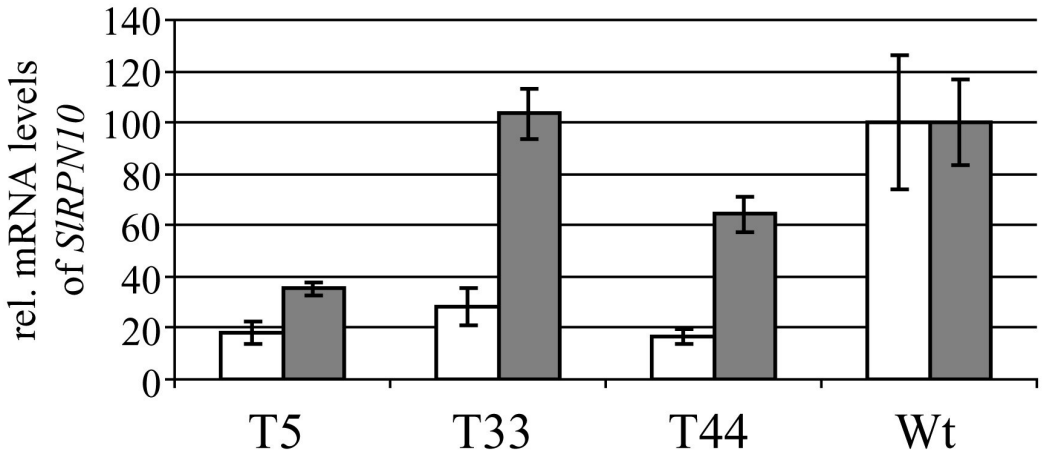


Fig. 1: Relative mRNA levels of a *SlRPN10* in leaf (white) and unpollinated ovary (grey). The mRNA levels in all three independent RNAi-lines are lower in leaf as compared to wild type (Wt). Lines T5 and T44 have lower mRNA levels in ovary as compared to wild type.

Figure 2A shows the phenotype of three months old wild type and T5 plants. Two of the most pronounced phenotypic characteristic of the *rpn10-1* mutant in Arabidopsis are yellowing of the leaves and reduced growth (Smalle *et al.* 2003). Both characteristics can be observed in the tomato RNAi-lines. Lines T5 and T44 displayed a size reduction; the plants are more slender and stop growing earlier, approximately after five months. The size reduction is also visible in the leaf size (Fig. 2B). Yellowing of the leaves started at the edges and was observed in all three lines (Fig. 2B). A slight but significant reduction in chlorophyll content of 15% was also observed in the young leaves of the transgenic lines as compared to

wild type (data not shown). To determine the ABA sensitivity we performed a root assay (Fig. 2C). Wild-type root growth is reduced by 5µ*M* and 10µ*M* ABA and hardly any growth was observed on 40µ*M*. In line T5 a significantly stronger reduction in root growth was observed on 5µ*M* and 10µ*M* ABA. The response of line T33 was similar to wild type on 5µ*M* ABA, but T33 responded significantly stronger to 10µ*M* ABA. In line T44 no significant difference, as compared to wild type, was observed.

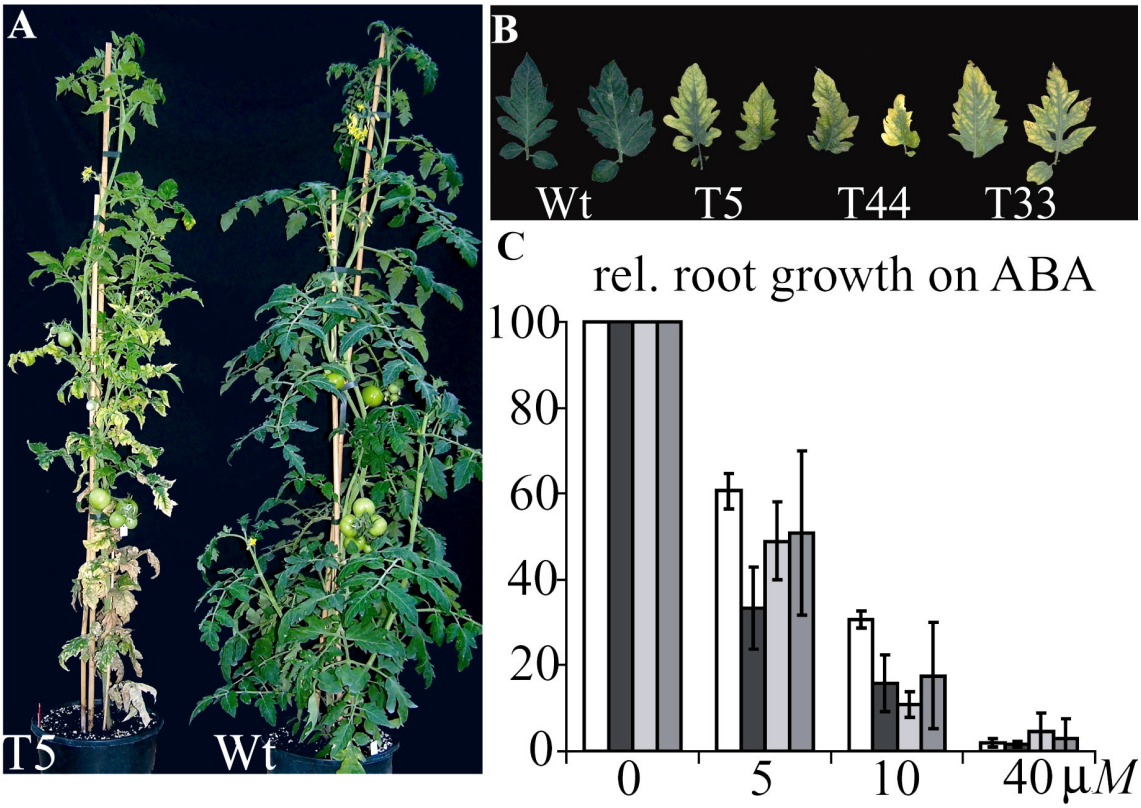


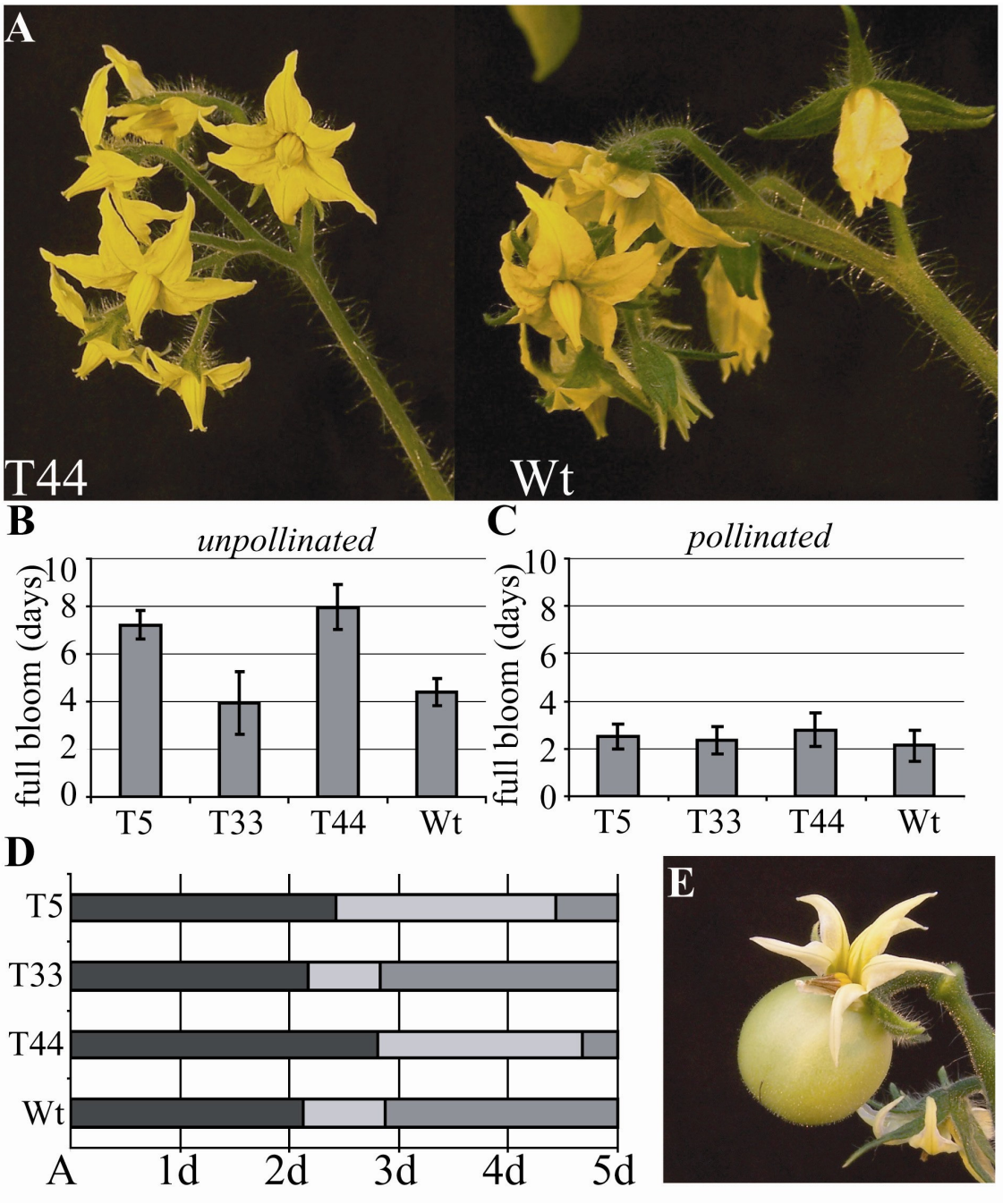
Fig. 2: A) Transgenic plants are more slender and more yellow than wild type. **B)** Leaves of transgenic lines T5, T33 and T44 show premature chlorosis, whereas wild-type leaves (Wt) are dark green. **C)** Relative root growth is more reduced on 5µM and 10µM ABA in T5 (black bars) and more reduced on 10µM ABA in T33 (light grey bars) than wild type (white bars), but not significantly different in T44 (dark grey bars). Averages of triplicate measurements are shown with SE.

Flower development

We further analyzed the flowers of the *SlRPN10*-silenced lines. No differences were detected in the growth and development of the flower buds from 2 mm to mature flowers. However, we noticed that most flowers on an inflorescence of the *SlRPN10*-silenced lines were in a similar stage (fully opened). On the contrary, wild type inflorescences have typically flowers at different stages of development; two to four fully opened flowers, several closed flowers and several flower buds (Fig. 3A). We therefore measured how long an emasculated flower remained fully opened if it was not pollinated (Fig. 3B). The transgenic lines T5 and T44 have flowers which were open until seven or eight days after anthesis (DAA), while the flowers of wild type and line T33 were on average open until four DAA. The increased full bloom phase of the flower is correlated with reduced mRNA level of the *SlRPN10* in ovaries. Because flower senescence is induced by pollination, we also measured how long flowers remain open after pollination with wild type pollen (Fig. 3C). Flowers from all four lines remained fully open approximately two and a half day after pollination (DAP), no significant differences between lines could be found. However, although the flowers of line T5 and T44 started closing at a similar time as wild type, further flower senescence progressed more slowly. The corolla did not close nor abscize as quickly as in wild type and flowers were half closed for a longer period (Fig. 3D). The corolla of some transgenic flowers even stayed attached after fruit formation (Fig. 3E).

Next page:

Fig. 3: A) Most flowers of an inflorescence in transgenic lines T5 and T44 are in a fully opened stage, while wild type (Wt) inflorescences have flowers at different stages of development (buds, fully opened and closed flowers). **B)** In T5 and T44 unpollinated flowers stay significantly longer fully opened as compared to wild type and T33. **C)** After pollination no significant differences in the length of the fully opened flower stage between wild-type and transgenic flowers can be found. **D)** Senescence in T5 and T44 progressed more slowly after pollination than in Wt and T33, as the time period in which the flowers stay half closed is longer. Flowers were hand pollination at anthesis (A), average (n=10) time period is depicted. Fully opened flower stage (black), half closed (light grey), closed (dark grey). **E)** The corolla of some flower of T5 and T44 stays attached after the start of fruit formation.



Anther and ovary development

Flower senescence thus seems to be delayed and therefore the full bloom phase of the flower is prolonged in the transgenic lines with reduced mRNA levels of the *SlRPN10* in ovary. In order to establish if the delay in flower closure is caused by a delay in overall flower development in the transgenic lines, we examined the development of the anthers and ovary

more closely. Figure 4A and B show the mature anthers and mature pollen of *SlRPN10*-silenced lines, which looked similar to wild type (not shown). Also during development no differences were found. Moreover, a pollen germination assay confirmed that the pollen of mature anthers of recently opened RNAi-flowers were germinating, although slightly less than wild type (70% and 80% respectively).

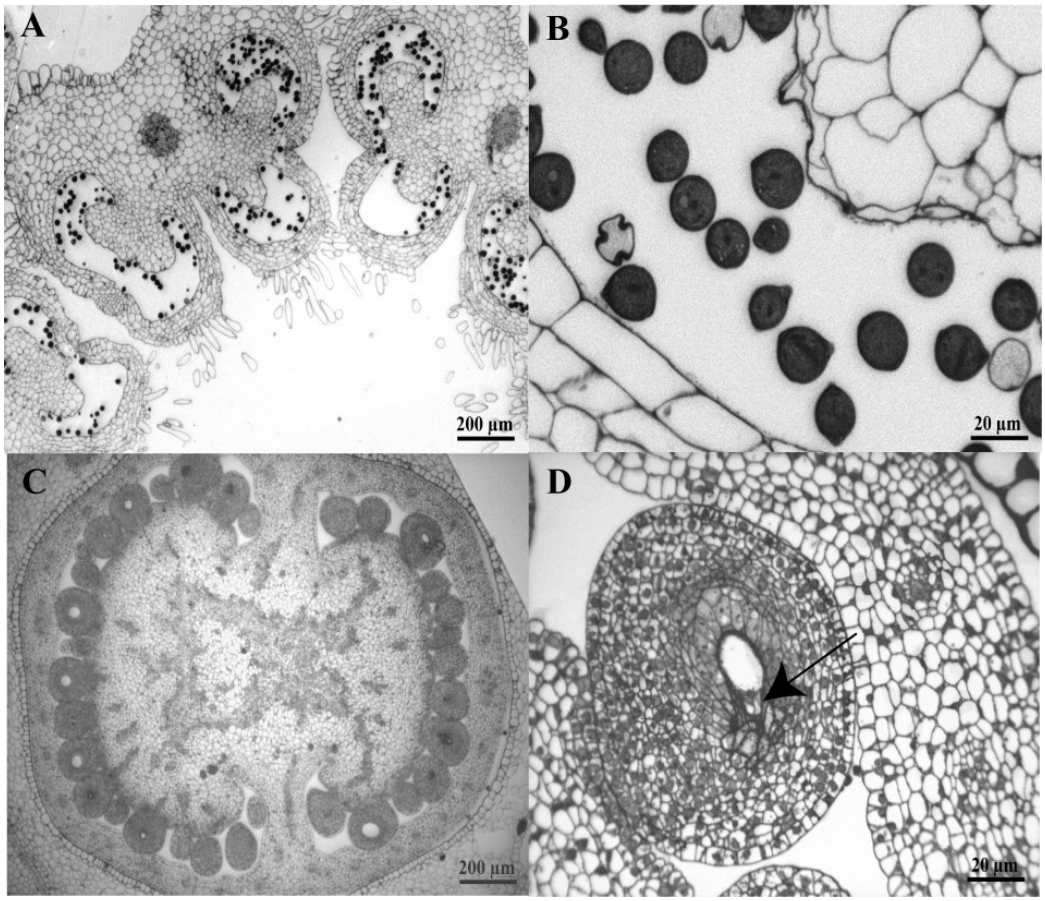


Fig. 4: Light microscopic pictures of ovary and anthers of a *SlRPN10*-RNAi line show anthers **(A)**, pollen **(B)**, ovary **(C)** and ovule **(D)** of a flower at anthesis, arrow is pointing to egg-cell and synergid cells. No differences with wild-type ovaries and anthers (not shown) were observed.

Ovary development also did not seem to be delayed. Mature ovaries of the *SlRPN10*-silenced lines looked similar to wild type (Fig. 4C), and the ovules were fully developed, the embryo sack with different cell types (egg-cell and synergid-cells) were present (Fig. 4D). Moreover, self-pollination led to seeded fruits. In older RNAi-flowers, four DAA and seven DAA, the anthers and ovaries were examined using field scanning electron microscopy (FESEM). No differences were observed between wild-type and *SlRPN10*-silenced plants. Four and seven DAA the pollen of both wild type and *SlRPN10*-silenced plants were unable to germinate and pollen germination rates were only two-three percent for both lines.

Fruit set

Although the ovules and ovary of the transgenic lines T5 and T44 look normal, we were interested to see if they set fruits equally well as wild type. Flowers that were pollinated with wild type pollen at anthesis (0 DAA) formed fruits in most of the cases in both wild-type and *SlRPN10*-silenced plants, fruit set percentages were 90% and 100% (Fig. 5). Flowers that were pollinated with fresh pollen four DAA also had fruit set percentages of 90% and 100%. Seven days after anthesis most of the wild-type flowers had lost their corolla and style, all were closed, and some had already abscized. Flowers that could still be pollinated hardly formed fruits. Although the flowers of the *SlRPN10*-silenced lines were fully opened in most cases only a very small percentage (10%) of the pollinated flowers set fruit (Fig. 5).

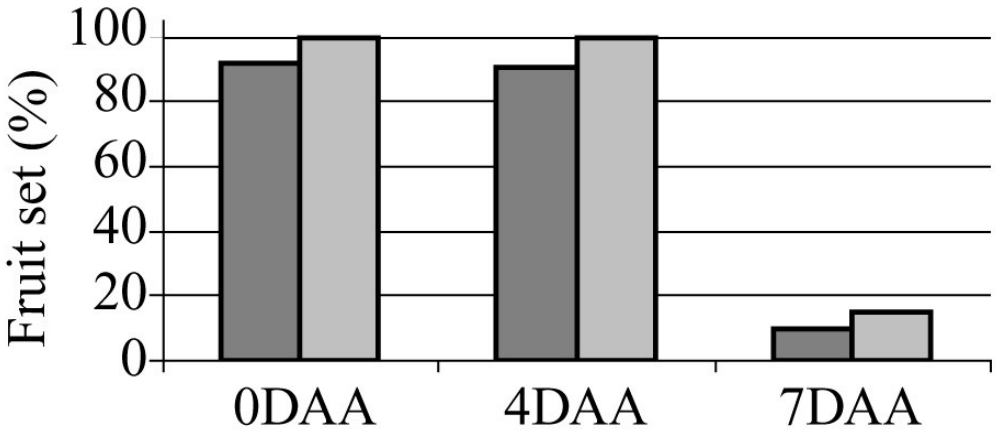


Fig. 5: Percentage of fruit set after hand pollination at anthesis (0 DAA), and four and seven days later (4 DAA and 7 DAA) is comparable in transgenic (dark grey) and wild-type (light grey) flowers.

Ethylene

Since flower senescence is often induced by ethylene, we measured the ethylene emission rate in emasculated flowers of the transgenic lines and wild type *in vivo* at different developmental stages. Transgenic flowers (lines T5 and T44) that were just starting to open (-2 DAA) had on average an ethylene emission rate of 0.1 nL/hr per flower, while wild-type flowers had an emission of on average 0.2 nL/hr per flower (Fig. 6A). Transgenic and wild type flowers were similar in size and weight. Flowers one DAA showed the same difference

between wild type and transgenic flowers as younger flowers did (Fig. 6B). Although there were some fluctuations in time no clear differences between day and night were found.

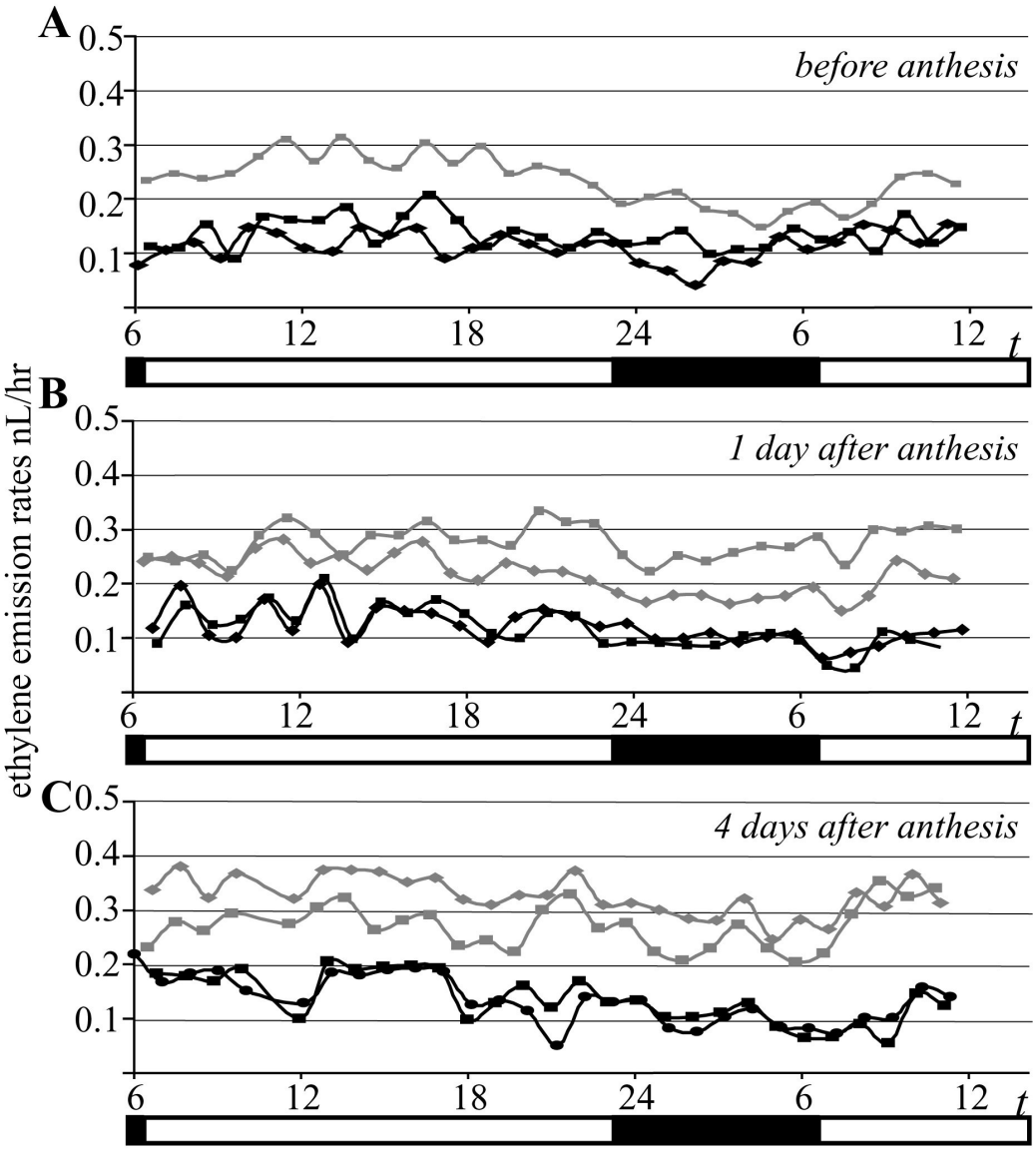


Fig. 6: Ethylene emission rates of individual flowers during a 36 hours time period.
A) When the flowers are starting to open, ethylene emission is lower in flowers of transgenic plants (black lines) than in flowers of wild-type plants (grey lines). **B)** Transgenic flowers one day after anthesis have lower ethylene emission rates than wild-type flowers. **C)** Even four days after anthesis (wild-type flowers start closing) ethylene emission rates in transgenic flowers are lower than in wild-type flowers. Figures show average ethylene measurements of typical flowers, SD values are at symbol level. Light-dark period (16 - 8 hrs) is shown in the black (darkness) - white (light) bar below each graph.

Eventually, we also measured ethylene emission in flowers four DAA. At that time wild type flowers were closing, while transgenic flowers were not. Again ethylene emission rates were lower in the transgenic flowers than in wild-type flowers (Fig. 6C). No peak in ethylene emission was measured throughout development. However, a higher ethylene emission rate was observed in flowers 4-5 DAA as compared to flowers precisely at anthesis. Ethylene emission increased from 0.1 nL/hr to 0.2 nL/hr in transgenic flowers and from 0.2 nL/hr to 0.35 nL/hr in wild type flowers.

The Arabidopsis *rpn10-1* mutant is ABA hypersensitive but also slightly auxin insensitive. Both changes in hormone sensitivity could be a possible cause of the reduced ethylene emission in the transgenic plants. Therefore we treated wild type and transgenic flowers with auxin and ABA while measuring ethylene emission. Figure 7 shows that 6µ*M* IAA induced a strong increase in ethylene emission to 1.25 nL/hr in wild type within three hours, while it only increased ethylene emission to 0.8 nL/hr in flowers of the transgenic *SLRPN10*-silenced line. In wild-type flowers a second increase of ethylene emission was visible nineteen hours after auxin application. In a separate experiment we added 10µ*M* ABA to wild-type flowers. We found no remarkable effect of ABA on ethylene emission rates. For reasons of comparison both experiments are depicted in the same figure 7.

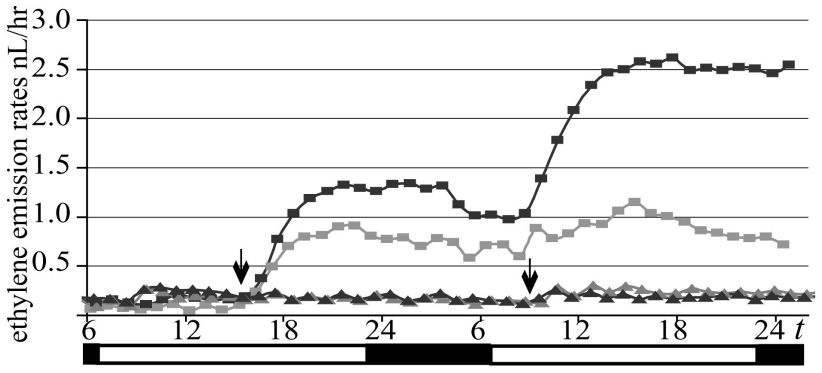


Fig. 7: Ethylene emission rates of single flowers after IAA (6µ*M*) and ABA (10µ*M*) application. First arrow indicates the IAA application to wild-type (black squares) and transgenic (grey squares) flowers. IAA induces an increase in ethylene emission rates. The increase seems to occur in two steps and is stronger in wild-type than in transgenic flowers. The second arrow indicates ABA application to wild type (grey triangles). ABA has no remarkable effect on ethylene evolution rates as compared to wild-type flowers treated with solvent only (black triangles). SD values are at symbol level.

Discussion

An RNAi approach to silence a *RPN10* homologue in tomato resulted in three transgenic lines which were silenced in leaf of which two were also silenced in unpollinated ovaries. Most likely a positional effect influences the expression of the transgene in the different tissues, this has also been reported e.g. in *PhEIN2* silenced plants of petunia (Shibuya *et al.* 2004). It is worth mentioning that only three transgenic lines were obtained via *Agrobacterium tumefaciens*-mediated transformation and tomato tissue culture, while three extensive and independent transformation-experiments were performed. As mentioned by Smalle *et al.* (2003) the Arabidopsis *rpn10-1* mutant exhibited decreased cytokinin sensitivity in shoots and roots, and reduced auxin sensitivity in the roots. Most likely this was also the case in tomato and could have negatively influenced the survival rate of the transgenic plants treated with hormones during the tissue culture process.

The vegetative phenotype of the Arabidopsis *rpn10-1* mutant and the *SlRPN10*-silenced lines in tomato are largely comparable. In both Arabidopsis and our transgenic tomato lines premature chlorosis was found. It has been shown that premature chlorosis also occurred in ABA over-producing plants, which were obtained by over expressing the *LeNCED1* gene under the *RbcS3C* promoter (Tung *et al.* 2008). Additionally we observed a growth reduction in stature and leaf size in our transgenic plants, this was also observed in Arabidopsis *rpn10-1* mutants. Both characteristics are suggestive of ABA hypersensitivity. Thus, it seems most likely that TC173550 is the functional orthologue of Arabidopsis *RPN10* (NM120024). However, unfortunately no perfect correlation between *SlRPN10*-silencing and increased ABA sensitivity in roots was observed. One should keep in mind that the seedlings used for this test were a mixture of heterozygous, homozygous and wild type plants. Therefore the presence of wild-type seedlings could have masked the results of the transgenic plants.

Further phenotypic examination of the *SlRPN10*-silenced plants revealed that the unpollinated flowers senesced later than wild type, indicating that the signaling route leading to the onset of petal senescence in absence of pollination include the action of the proteasome and of subunit RPN10. After pollination the flowers of the *SLRPN10*-silenced lines started petal closure at similar time as wild type, but the closure process progressed more slowly. Most likely, the senescence process itself also involves the action of the proteasome and RPN10. It has indeed been shown that petal senescence is associated with enhanced actitvity of the ubiquitin-proteasome route of protein degradation and that proteasome action was required for senescence in Iris and Petunia (Van Doorn and Woltering 2008).

The delayed flower senescence we observed was not caused by an overall delay in flower development in the *SlRPN10*-silenced lines. We could establish by light microscopy that pollen and embryo-sack developed normally and timely in the transgenic *SlRPN10*-silenced lines. Additionally, no differences were found in the percentage of fruit set in the transgenic neither at anthesis nor several days after anthesis. Petal senescence in this case is thus not a measure for the state of the ovary, which is clearly not receptive anymore even if the flowers of the *SlRPN10*-silenced lines are still opened.

The stimulating effect of the hormone ethylene on flower senescence has been proven for instance by ethylene insensitive transgenic plants of Petunia and tobacco, which had delayed flower senescence (Shibuya *et al.* 2004; Yang *et al.* 2008). Some species however have ethylene insensitive flower senescence, but Llop-Tous *et al.* (2000) showed that in tomato pollination induced an increase in ethylene synthesis, causing flower senescence. Moreover, the *never-rip*e tomato mutant, which is ethylene-insensitive, showed delayed flower senescence both after pollination and without pollination (Llop-Tous *et al.* 2000). These observations indicate that ethylene is involved in flower senescence in tomato. Reduced *SlRPN10* expression resulted in reduced ethylene emission in unpollinated flower. This might indicate that SlRPN10 is normally stimulating ethylene production, possibly by degrading proteins that inhibit ethylene production. This could be the cause of reduced flower senescence.

The reduced ethylene emission in the *SlRPN10*-silenced lines could also be caused by the ABA hypersensitivity in these plants. An inhibitory function for ABA on ethylene production has been reported in shoot growth of tomato and Arabidopsis (LeNoble *et al.* 2004; Sharp *et al.* 2000). However, we found that ABA application to wild type flowers did not reduce ethylene emission. In carnation it has been reported that exogenous ABA application to the flower induces an ethylene peak resulting in flower senescence (Shibuya *et al.* 2000; Nukui *et al.* 2004). Moreover, flower senescence in ABA-deficient lines, such as *notabilis, flacca*, and *not/flc* double mutants, was not different from wild type (own observations). It thus seems unlikely that ABA is inhibiting ethylene emission in mature flowers, although this might be concentration dependent. The reduced ethylene emission in the *SlRPN10*-silenced plants could also be explained by a reduced auxin sensitivity, which has been reported in the *rpn10-1* mutant in Arabidopsis (Smalle *et al.* 2003). Indeed, we measured that IAA application to wild-type tomato flowers induced an ethylene peak and that this peak was lower in flowers of the *SlRPN10*-silenced lines, suggesting that their auxin sensitivity is

reduced. In carnation IAA application also lead to increased ethylene emission (Shibuya *et al.* 2000; Nukui *et al.* 2004). Reduced auxin sensitivity in the *SlRPN10*-silenced lines thus might reduce the ethylene emission in unpollinated flowers, which possibly is the cause of reduced flower senescence. However, auxin is a positive stimulator of fruit set in the signaling cascade after pollination. In unpollinated ovaries auxin levels, and the gene-expression of auxin signaling genes, are low (Gillaspy *et al.* 1993; Vriezen *et al.* 2008) and an increase in auxin in the absence of pollination leads to parthenocarpic fruits. Increased auxin levels or auxin sensitivity therefore seems to be an unlikely mechanism to regulate petal senescence in the absence of pollination. Nevertheless, different hormone concentrations might differentiate between fruit induction and petal senescence without fruit induction. Altogether, the hormonal interactions between ethylene, auxin and ABA leading to flowers senescence are an intricated network that still requires further research.

Acknowledgement

We would like to thank Dr. S te Lintel Hekkert (SensorSense BV, Nijmegen) for giving us the opportunity and assistance to measure ethylene *in vivo*.

Materials and Methods

Plant growth-conditions

Tomato plants (*Solanum lycopersicum* L. cv. Moneymaker from Enza Zaden, Enkhuizen, The Netherlands) were grown under greenhouse conditions from March to October under 16/8h day-night rhythm. Supplementary lights (600 Watt high pressure sodium lights) turned on below 200 W/m^2 and turned off above 300 W/m^2. Temperature was kept above 20°C during the light period and 17°C during the dark period with the PRIVA Integro versie 724 system. Plants were watered daily and given fertilizer weekly. Leaf and ovary tissues were dissected form adult tomato plants between 11.00 hrs and 13.00 hrs and directly frozen in liquid nitrogen.

Production of transgenic plants

To generate transgenic *SlRPN10*-RNAi lines, a part of the *SlRPN10*-sequence (SGN-U216576, base pair 1183-1613) was PCR amplified and cloned in pDONR vector. Using Gateway cloning this part was cloned behind the *Cauliflower Mosaic Virus 35S* promoter in

the pK7GWIWG2(II) vector which also contains a *NOPALINE SYNTHASE* terminator. Transgenic plants were generated by *Agrobacterium tumefaciens*-mediated transformation and tissue culture as described in De Jong *et al.* (2008).

RNA isolation and cDNA synthesis

RNA was isolated with Trizol (Invitrogen, Carlsbad, CA, USA). Photometric RNA measurements were done to equilibrate the RNA concentrations of different samples. Equal amounts of RNA were DNAse treated with RNAse free DNAse (RQ1, Promega, Madison, USA). RNA (0.5 µg) was reverse transcribed (RT) using a cDNA synthesis kit (iScript^{tm}, Bio-rad Laboratories, Hercules, CA, USA) following protocol.

Real time quantitative RT-PCR

Real-time-quantitative RT-PCR (Q-PCR) primers for *SlRPN10* (5'-AATCCTTG CCTCACTTCCAG-3' and 5'- GTTCCTTATCATTGTCCTCATCC -3') were designed using a computer program (Beacon Designer Software, Premier Biosoft International, CA, USA). PCR reactions were performed in a 96-well thermocycler (Bio-Rad iCycler, Bio-rad laboratories) using a temperature program starting with 3 min at 95°C then 40 cycles consisting of 15 s at 95°C and 45 s at 57°C and finally the melting temperature of the amplified product was determined to verify the presence of a specific product. Five micro liter of 25-fold diluted cDNA was used per sample. Technical and biological replicates were always performed. Both Actin 2/7 (5'-GGACTCTGGTGATGGTGTTAG-3' and 5'-CCGTTCAGCAGTAGTGGTG-3') and Ubiquitin 7 (5'-CCCTGGCTGATTACAAC ATTC-3'and 5'-TGGTGTCAGTGGGTTCAATG-3') were used as internal control genes, to correct for difference in cDNA amounts. Dilluted DNase treated RNA was also included in the Q-PCR as a control for genomic DNA contamination. Mean values of biological repeats are depicted together with the SE.

Root assay

Seeds were sterilized in diluted bleach (4% hypochlorite) containing 0.1% tween, washed and sown in water containing 1µ*M* GA_3. After radicle emergence ten seeds of each line were placed on 1/2MS media containing either, 0, 5, 10, or 40 µ*M* ABA. The roots were

measured and the plates were placed in vertical position and grown in a growth chamber at 25°C under 16/8h day-night rhythm. Seven days after transfer the length of the root was measured again and average root growth was calculated as a percentage compared to 1/2MS media without ABA (100%). Experiments were carried out three times and averages plus SE are shown.

Flower senescence

Fifteen flower buds were labeled and scored every day in the following categories: opening, fully opened, half closed or closed. Eventually the number of days the flowers were fully opened was calculated and half a day was added for the last day opening and/or the first day half closed. The number of days the flowers were half closed was also calculated. The average time period the flowers are fully opened or half closed was calculated per line. Averages are depicted with SD.

Cryo-Field-Emission scanning electron microscope FESEM

For each line, two transversal slices of two anther cones of older flower (four and seven days after first full bloom) were placed on the microscope disc in the carbon paste and rapidly frozen in liquid nitrogen. The frozen tissue was transferred to the vacuum chamber of the Cryo-Field-Emission scanning electron microscope (FESEM, JEOL-6330). After 4 minutes sublimation at -90°C, the samples were sputtered with gold/platinum for 40 s at -140°C, and examined in the microscope at -140 °C. Photographs were made at different magnifications, varying from 30 until 500 times. FESEM was performed at the general instrumentarium of the science faculty (GI, Radboud university Nijmegen)

Pollen germination assay

Three anther cones of mature or older flowers (four and seven days after first full bloom) were cut and 300µL of pollen germination media containing 20mM Mes, pH 6.0, 3 mM $Ca(NO_3)_2$, 1mM KCl, 0.8 mM $MgSO_4$, 1.6 mM boric acid, 2.5 % (w/v) Suc, and 24 % (w/v) polyethylene glycol 4000, was added. After intensive vortexing, to release the pollen, they were incubated for three hours at room temperature. Pollen-germination was examined under a light microscope at 10x objective. A minimum of 100 pollen grains was scored (normally in 10 frames). Six anther-cones per line were used. Mean germination percentages and SD were calculated.

Fruit set

Wild type and transgenic flowers were emasculated 3 days before anthesis (-3 DAA). Flowers were pollinated at anthesis (0 DAA) and 4 and 7 DAA. One week after pollination fruit set was scored. When ovaries were larger than 3 mm they were scored positively as fruits. Percentage of fruit set was calculated from ten flowers.

Ethylene measurement

Ethylene production was measured in real time with a sensitive laser-based ethylene detector (type ETD-300, Sensor Sense B.V., Nijmegen, the Netherlands) in combination with a gas handling system. Both the ETD-300 and the valve control box are operated fully automatically and can perform continuous measurements for periods of several days until weeks (Salman *et al.* 2008; De Grauwe *et al.* 2008). The ETD-300 is a state-of-the art ethylene detector based on laser photoacoustic spectroscopy (Cristescu *et al.* 2008) that is able to detect on-line about 300 pptv (pptv = parts-per-trillion volume, $1{:}10^{12}$) of ethylene within 5 seconds. The gas handling was performed by a valve control box (type VC-6, Sensor Sense B.V., Nijmegen, the Netherlands), designed for measuring up to 6 sampling cuvettes per experiment. The valve control box allowed continuous constant air flow of 1 L h^{-1} through the cuvettes and automated sampling of ethylene production to the ETD-300 alternately, in succession of 10 min for each cuvette. In this study six glass cuvettes (10 ml volume) were used per experiment, from which five were containing a single tomato flower and one reference without flower. The air was sampled from the lab and passed through a platinum-based catalyzer (Sensor Sense B.V., Nijmegen, the Netherlands) to remove traces of external ethylene or other hydrocarbons. A scrubber with KOH was used to reduce the CO_2 concentrations to less than 1 ppm, and a tube with $CaCl_2$ was placed directly after this scrubber in order to decrease the water content in the gas flow. Each experiment was repeated giving similar results.

Chapter 5

Changing ABA sensitivity through over-expression and co-suppression of *SlPP2C1,* a homologue of *AtABI1*

L. Nitsch, A. Derkx, C. Mariani and W.H. Vriezen

Abstract

AtABI1 and *AtABI2* encode negative regulators of the abscisic acid (ABA) response. Here we report of the isolation of a homologue of *AtABI1* and *AtABI2* from tomato; *SlPP2C1*, which is differentially expressed during fruit set. Over-expression and co-suppression of *SlPP2C1* in tomato resulted in ABA-insensitive and ABA-hypersensitive tomato plants. Root growth, seed germination and drought responses were altered in the transgenic lines in a way that indicates that *SlPP2C1* encodes a negative regulator of the ABA response in tomato as well. Transgenic plants co-suppressing *SlPP2C1* provide interesting tools to study the role of ABA during fruit set in tomato.

Introduction

The phytohormone abscisic acid (ABA) is important for the regulation of abiotic stress responses and seed dormancy. Screens for mutants with altered abiotic stress responses or seed dormancy have been used frequently and resulted in the identification of genes important for ABA biosynthesis and ABA signal transduction. Via such a screen the Arabidopsis ABA-insensitive mutants *abi1-1* and *abi2-1* have been identified (Koornneef *et al.* 1984). Cloning and characterization of the *AtABI1* gene revealed that it encodes a serine/threonine protein phosphatase type 2C (PP2C, Leung *et al.* 1994; Meyer *et al.* 1994). *AtABI2* also encodes a protein phosphatase type 2C. a*bi1-1* and *abi2-1* mutants carry mutations in the *AtABI1* and *AtABI2* genes, which result in identical Gly-to-Asp substitutions at equivalent positions (Leung *et al.* 1997). Both mutants were shown to have reduced phosphatase activity (Bertauche *et al.* 1996; Leung *et al.* 1997), which would suggest that AtABI1 and AtABI2 are positive regulators of ABA sensitivity. However, constitutive over-expression of *AtABI1* inhibited ABA action in maize protoplast, and reduction-of-function mutants of *AtABI1* and *AtABI2* were shown to have hypersensitive responses to ABA (Scheen 1998; Gosti *et al.* 1999; Merlot *et al.* 2001). Altogether, it was therefore concluded that AtABI1 and AtABI2 are negative regulators of the ABA response. The exact mechanism by which the mutations in *abi1-1* and *abi2-1* induce ABA insensitivity is still unknown, although it might be related to the preferential nuclear localization of the mutated proteins (Moes *et al.* 2008). AtABI1 and AtABI2 are important for seed dormancy but also for seedling growth and regulation of stomatal aperture, suggesting that these proteins act before major branch points that control tissue-specific ABA signaling cascades (Leung *et al*. 1997)

Seventy-six *PP2C*s have been identified in Arabidopsis, of which one group, comprized of nine genes, has been associated with ABA signal transduction (Schweighofer *et al.* 2004). Several of the Arabidopsis genes belonging to this group were also found to encode negative regulators of the ABA response, among them are *AtP2C-HA* (Rodriguez *et al.* 1998), *AtHAB1* (Saez *et al.* 2004), and *AtPP2CA* (Kuhn *et al.* 2005). We have previously identified a putative tomato (*Solanum lycopersicum* L.) homologue of *AtABI1* an *AtABI2*, designated *SlPP2C1*, in a cDNA-AFLP designed to screen for genes differentially expressed during fruit set (Vriezen *et al.* 2008). This gene was relatively highly expressed in unpollinated ovaries and less expressed in pollinated ovaries. Additionally, we found more ABA related genes with a similar expression pattern as *SlPP2C1*, suggesting that ABA signaling and responses are relatively high in mature unpollinated ovaries and decrease after pollination (Vriezen *et al.* 2008). Moreover, we found that ABA levels were high in unpollinated ovaries and low after pollination (chapter 2). These data together suggest a role for ABA in mature ovaries or during fruit set. The tomato *AtABI1*-homologue might be important for the signal transduction of ABA in this process. Moreover, plants with changed sensitivity for ABA might provide the necessary tools to study the function of ABA in mature ovaries and during fruit set. We therefore set out to further characterize the tomato *SlPP2C1* gene by generating and analyzing transgenic tomato plants in which *SlPP2C1* is silenced or over expressed. Here we report that *SlPP2C1* is involved in ABA signaling and that over-expression or co-suppression leads to ABA- hypersensitive and ABA- insensitive tomato plants.

Results

Expression and phylogeny of *SlPP2C1*

The *SlPP2C1* gene is differentially expressed during fruit set (Vriezen *et al.* 2008). Figure 1A shows that *SlPP2C1* is expressed highest in the pericarp of unpollinated ovaries and lower after pollination and GA_3-treatment. The expression in ovules/placenta does not seem to change. We confirmed the expression of *SlPP2C1* within the tissues of the tomato ovary by quantitative RT-PCR. Again it was observed that the mRNA levels of *SlPP2C1* are higher in the pericarp than in the ovules or placenta in control tissue. In the pericarp, but also in the placenta, a lower mRNA level of *SlPP2C1* was found three days after pollination. In ovules mRNA levels did not change (Fig. 1B). Figure 1C shows that the *SlPP2C1* gene is expressed in mature unpollinated ovaries at anthesis at a relatively high level compared to

vegetative tissues such as leaf, root and hypocotyl. In flower buds and in ovaries three days before anthesis the *SlPP2C1* mRNA level is comparable to leaf, and it is much lower than in ovaries at anthesis (control, Ct). Three days after pollination *SlPP2C1* mRNA levels in the ovary were reduced to approximately 50% of the level in unpollinated ones.

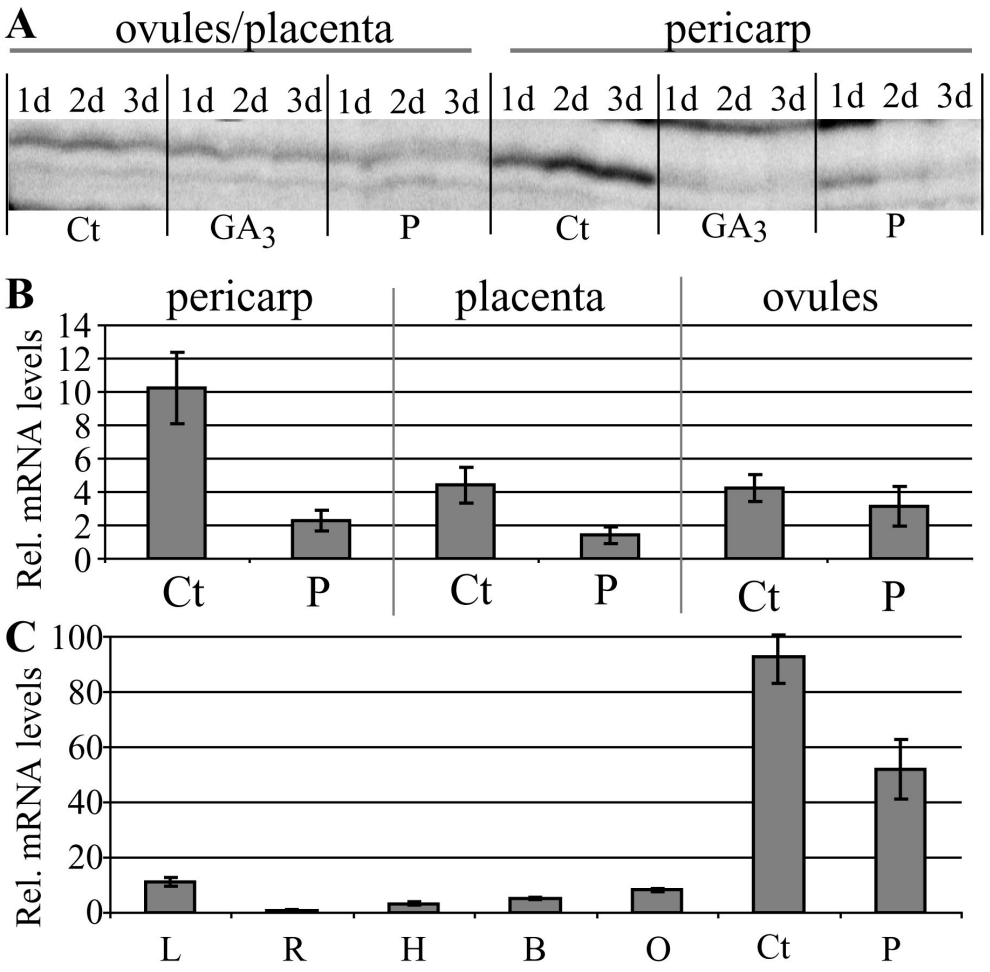


Fig. 1: A) cDNA-AFLP indicates higher levels of *SlPP2C1* in the pericarp of untreated ovaries (Ct) as compared to the pericarp of pollinated (P) or GA_3 treated (GA_3) ovaries. In ovules/placenta no obvious changes in mRNA levels of *SlPP2C1* are visible. **B)** Relative mRNA levels of *SlPP2C1* are highest in the pericarp of unpollinated ovaries (Ct) and lower three days after pollination (P) in pericarp and placenta. No changes in mRNA levels in ovules are observed after pollination. **C)** Relative mRNA levels of *SlPP2C1* are higher in unpollinated mature ovaries (Ct) as in leaf (L), root (R), hypocotyls (H), flower bud (B) and ovary three days before anthesis (O). Three days after pollination (P) a decrease in mRNA levels of 50% is visible in ovaries as compared to control (Ct). Mean values of biological replicas and SE are shown and the highest values are set to 100.

In order to get an overview of the ABA-associated *PP2C* family in tomato we performed an Expressed Sequence Tags (ESTs) search in the Gene Indices database (http://compbio.dfci.harvard.edu/tgi). We found eight ESTs, designated as *SlPP2C1* until *SlPP2C8*, with homology to one of the Arabidopsis *PP2C* genes associated with ABA; for homology percentages see table 1 in materials and methods. Most of the tomato EST sequences we found seem to be full length, only the *SlPP2C2* sequence lacks the 5'-end, this sequence was therefore excluded from the neighbor-joining analysis, other putative ABA associated tomato *PP2C*s and all nine *Arabidopsis PP2C*s known to be involved in ABA signaling were included. The sequence of a PP2C unrelated to ABA (KAPP, At5g19280) was used as an out-group (Fig. 2). The neighbor-joining analysis identified two main clades in which both amino-acid sequences of Arabidopsis and tomato group together. SlPP2C1, which was previously identified as transcript in our cDNA-AFLP, falls into the second clade which includes five Arabidopsis PP2Cs (among others AtPP2CA) and four other putative tomato PP2Cs (SlPP2C4, -6, -7, -8). Because *SlPP2C1* was differentially expressed during fruit set the coding region of this gene was used to make an over-expression construct that was transformed to tomato.

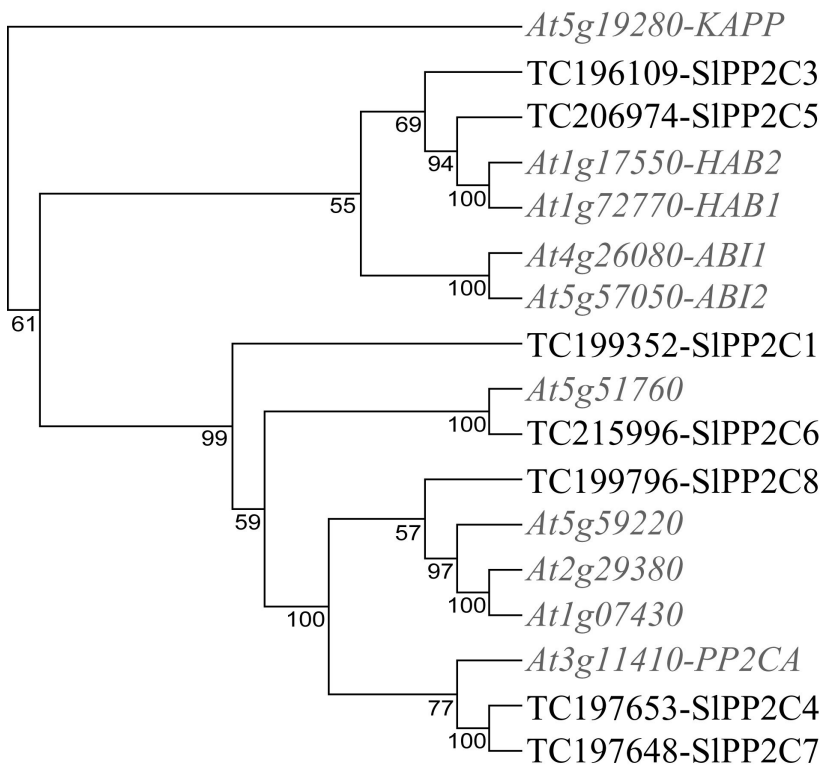


Fig. 2: Neighbor-joining tree of Arabidopsis (grey italic) and tomato (black roman) amino acid sequences of putative PP2Cs related to ABA. Two major clades were identified, each containing sequences of both Arabidopsis and tomato. SlPP2C1 falls into a clade together with AtPP2CA, At5g51760, At5g59220, At2g29380, At1g07430 and SlPP2C4,6,7,8. Bootstrap values (n=100) are indicated at junctions.

Functional analysis of *SlPP2C1*

The over-expression approach resulted in three lines with on average 25-fold higher mRNA levels of *SlPP2C1* in leaves ($T6_{OE}$, $T34_{OE}$ and $T55_{OE}$, Fig. 3A). In pollinated ovaries the *SlPP2C1* mRNA levels of these lines were not higher as in wild type (Fig. 3B). On the contrary, $T6_{OE}$ and $T34_{OE}$ displayed a strong reduction in *SlPP2C1* mRNA levels as compared to wild type. $T55_{OE}$ had mRNA levels similar to wild type in pollinated ovaries. The *SlPP2C1* mRNA levels in unpollinated ovaries of these transgenic lines were comparable to the mRNA levels in pollinated ovaries and are not depicted here. Additionally, two lines ($T12_{CS}$ and $T35_{CS}$) were obtained that had lower *SlPP2C1* mRNA levels in leaves and unpollinated ovaries, as compared to wild type (Fig 3C). Apparently over-expression led to co-suppression in these lines. Line T18 has mRNA levels that are comparable to wild type in both tissues.

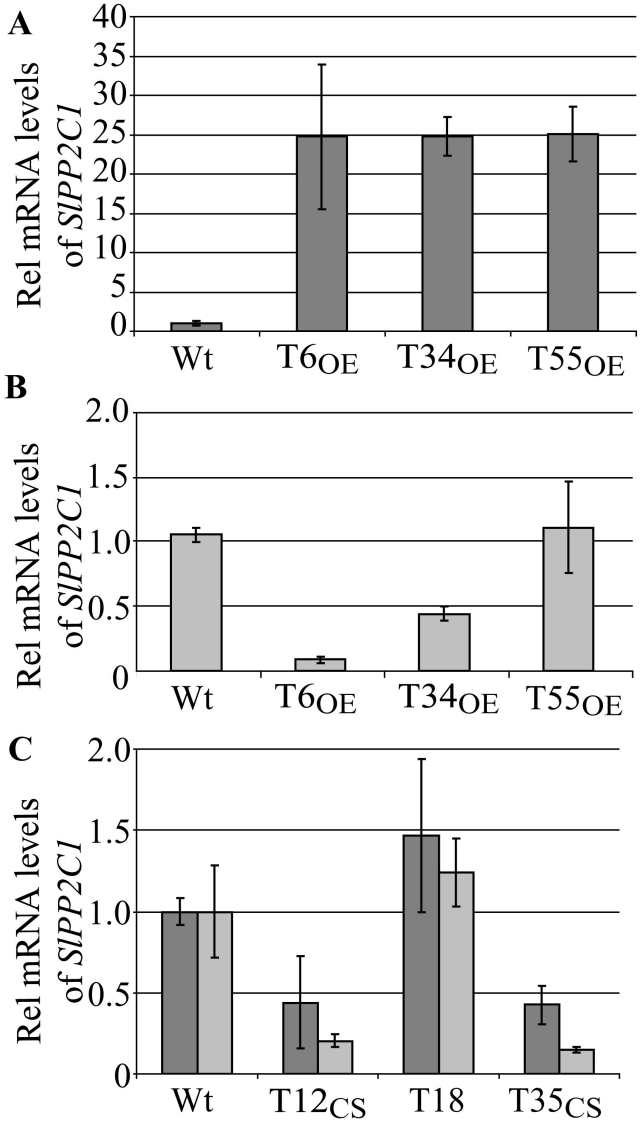


Fig. 3: A) Relative mRNA levels of *SlPP2C1* in leaf (dark grey bars) of transgenic lines ($T6_{OE}$, $T34_{OE}$, $T55_{OE}$) are 25-fold higher as compared to wild type (Wt). **B)** Relative mRNA levels of *SlPP2C1* in pollinated ovaries (light grey bars) in $T55_{OE}$ are similar to wild type, while the levels in $T6_{OE}$ and $T34_{OE}$ are lower as compared to wild type. **C)** Relative mRNA levels in transgenic lines $T12_{CS}$ and $T35_{CS}$ are lower in both leaves (dark grey bars) and unpollinated ovaries (light grey bars) as compared to wild type and T18. Mean values of biological replicas are shown with SE, wild type levels were set to one.

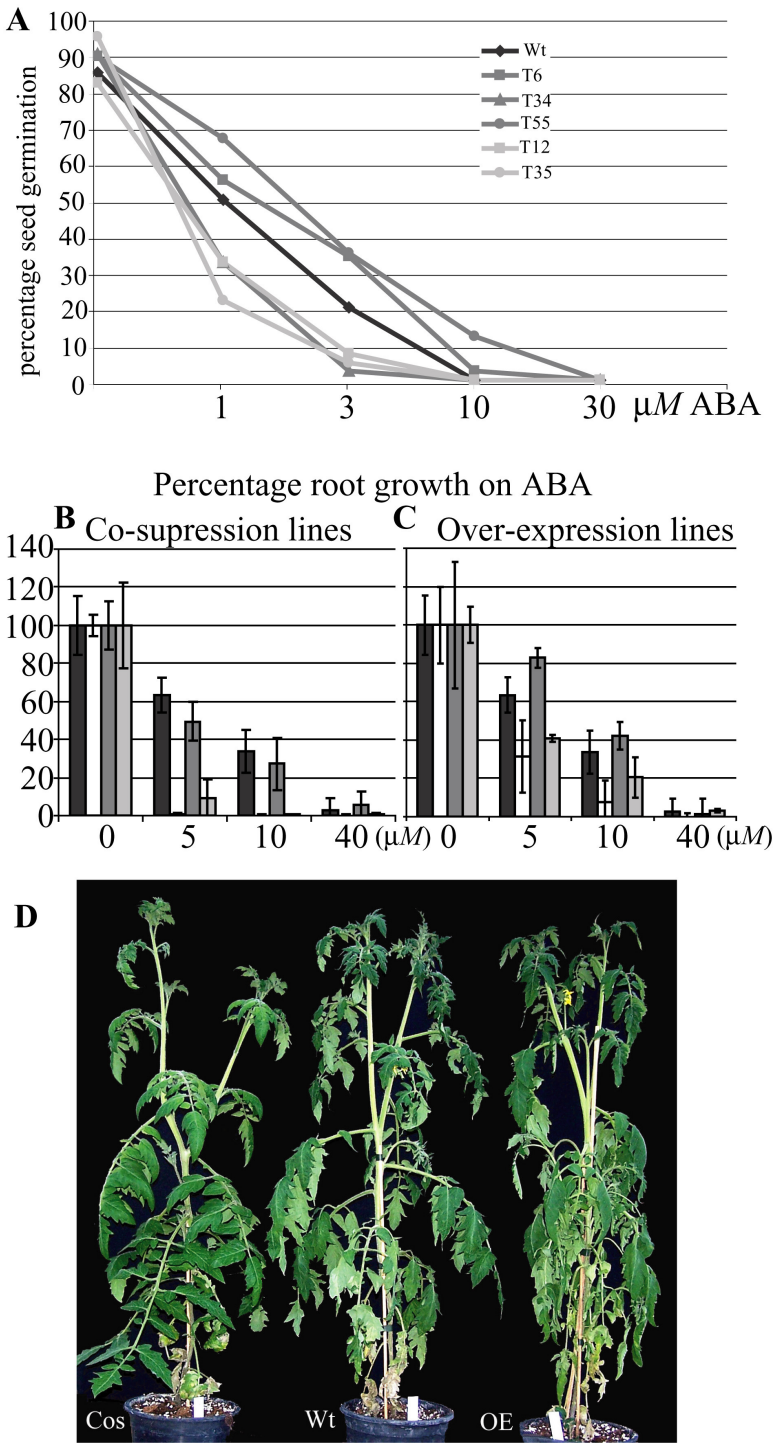


Fig. 4: A) Seed germination is inhibited less by ABA in the over-expression lines $T6_{OE}$ and $T55_{OE}$ and more in the co-suppression lines $T12_{CS}$ and $T35_{CS}$ as compared to wild type (Wt). Line $T34_{OE}$ behaves atypically. Dark grey lines represent over-expression lines, light grey lines represent co-suppression lines and the black line represents wild type. **B)** Root growth is inhibited more by ABA in the co-suppression lines $T12_{CS}$ (white) and $T35_{CS}$ (light grey) as compared to T18 (dark grey) and wild type (black). **C)** Root growth is inhibited less by ABA in the over-expression line $T34_{OE}$ (dark grey), but more in $T6_{OE}$ (white) and $T55_{OE}$ (light grey), as compared to wild type (black). Mean root growth percentage is depicted with SD. **D)** Nine days after the start of water with-holding co-suppression lines are not wilted at all, while wild type shows slight wilting and over-expression lines show more severe wilting.

Phenotypic characterization of the transgenic lines revealed changed sensitivity for ABA. Figure 4A, shows the percentage of seed germination on media containing different concentrations of ABA. The lines $T12_{CS}$ and $T35_{CS}$ have lower germination percentages on 1μ*M* and 3μ*M* ABA, while the lines $T6_{OE}$ and $T55_{OE}$ have slightly higher germination percentages as wild type. Line $T34_{OE}$, which is an over-expression line in leaves, behaved differently from the other over-expression lines and showed lower seed germination on ABA as compared to wild type. Root growth after germination is reduced by ABA in wild type. In figure 4B it is visible that root growth in $T12_{CS}$ and $T35_{CS}$ was inhibited more strongly than in wild type or T18. Line $T34_{OE}$ was less sensitive to root growth inhibition by ABA than wild type (Fig. 4C). On the contrary, root growth in lines $T6_{OE}$ and $T55_{OE}$ was more sensitive to ABA. We also observed faster wilting in plants of all the over-expression lines compared to wild type. By contrast, plants of the co-suppression lines displayed less wilting than wild type during the ten days period of water with-holding (Fig. 4D).

Discussion

Our neighbor-joining analysis identified two major clades which were comparable to the ones which were identified by Schweighofer *et al.* (2004) based on the Arabidopsis sequences alone. The tomato sequences were spread over these different clades. The first big clade contains SlPP2C3 and SlPP2C5 together with AtABI1, AtABI2, HAB1 and HAB2. These Arabidopsis proteins were all proven to be involved in ABA signaling (Koornneef *et al.* 1984; Rodriguez *et al.* 1998; Saez *et al.* 2004). SlPP2C3 and SlPP2C5 thus seem to be the closest homologues of AtABI1 and AtABI2. The second major clade was divided into several sub-clades of which one includes SlPP2C8, At5g59220, At2g29380, and At1g07430. Null mutations in the Arabidopsis genes encoding for the latter proteins did not reveal any changes in ABA sensitivity (Yoshida *et al.* 2006), indicating that perhaps these proteins, and thus also SlPP2C8, are not involved in ABA signaling or are highly redundant. The two other sub-clades contained AtPP2CA and At5g51760 which were both proven to be involved in ABA signaling (Kuhn *et al.* 2005; Nishimura *et al.* 2007). SlPP2C1 falls into this second big clade but is not further grouped into a sub-clade.

SlPP2C1 was differentially expressed in the ovary during fruit set. It is known that ABA induces the expression of *AtABI1* and other ABA-related *PP2C*s, thereby stimulating a negative feedback loop in its own signaling cascade (Kuhn *et al.* 2005; Leung *et al.* 1997;

Rodriguez *et al.* 1998). Therefore, it seems likely that *SlPP2C1* mRNA levels are high in unpollinated ovaries due to the high ABA content in this tissue (chapter 2). After pollination the ABA content decreases (chapter 2), and probably this is the cause of the decrease in *SlPP2C1* mRNA levels after pollination. It is however remarkable that there is no reduction in the mRNA level of *SlPP2C1* in the ovules. A gene encoding for an ABA catabolic enzyme, *SlCYP707A1*, is specifically higher expressed in ovules after pollination (chapter 2). This suggests that ABA levels decrease especially there, and therefore also the *SlPP2C1* mRNA level would be expected to decrease, but it does not. Possibly, the *SlPP2C1* mRNA level in ovules of unpollinated ovaries is already relatively low, only 35% of the mRNA level in pericarp, and a further decrease might not be under control of the ABA level. This might indicate that the ovules have a relatively high ABA sensitivity before pollination; after all low mRNA levels of *PP2C*-genes render plants ABA-hypersensitive.

Two of the transgenic lines harboring an over-expression construct had lower levels of *SlPP2C1* mRNA in both leaves and ovaries. RNA silencing by co-suppression is a well accepted phenomenon although the mechanism is not fully understood. It has been suggested that co-suppression is induced by hairpin-RNA transcripts from inverted-repeat transgene copies, resulting in siRNAs that are incorporated into the RNAi pathway (Tomita *et al.* 2004; Wang and Metzlaff 2005). Southern blot analysis (data not shown) revealed that in the two co-suppression lines of *SlPP2C1* multiple insertions were present, which might have resulted in a hairpin-like structure that could silence the *SlPP2C1* gene. The three lines with a 25-fold higher mRNA level of *SlPP2C1* in leaf did not have higher *SlPP2C* mRNA levels in ovaries. This might be partly explained by the relatively high mRNA level of the endogenous gene in ovaries, which is seven fold higher than in leaf. The contribution of the transgene to the total expression levels of *SlPP2C1* in ovaries might therefore have been very small. Remarkable is that in line $T6_{OE}$ and $T34_{OE}$ the mRNA level of *SlPP2C1* in ovary is even lower (10-50%) than in wild type. Tissue specific control of endogenous mRNA levels by a transgene has however been reported before. Tomita *et al*. (2004) showed that in the same plant the *NtFAD3* gene was co-suppressed in leaf but over-expressed in root, resulting in the equivalent phenotypes in leaves and roots. The mechanisms by which tissue specific regulation of co-suppression occurs are unknown, but the level of endogenous transcript seems to be important (Tomita *et al.* 2004).

The *AtABI1* and *AtABI2* genes from Arabidopsis code for negative regulators of ABA signaling and it has been shown that reduction-of-function mutants in these genes display

higher ABA sensitivity (Gosti *et al.* 1999; Merlot *et al.* 2001). Accordingly, the two tomato transgenic lines with reduced mRNA levels in leaf and ovary also displayed ABA-hypersensitive responses during seed germination, root growth and water stress. This indicates that the *SlPP2C1* gene is a negative regulator of the ABA signaling cascade in tomato as well. Moreover, the three transgenic lines with relative high mRNA levels of *SlPP2C1* in leaf also wilted faster, indicative of ABA insensitivity. Together with the reduced ABA sensitivity of $T6_{OE}$ and $T55_{OE}$ in the seed germination assay, these data strongly suggest that *SlPP2C1* codes for a negative regulator of the ABA response. Interestingly, the response of $T6_{OE}$, $T34_{OE}$ and $T55_{OE}$ was not always as expected from an "over-expression" line. $T34_{OE}$ displayed an ABA hypersensitive phenotype in the seed germination assay, and $T6_{OE}$ and $T55_{OE}$ displayed a hypersensitive phenotype in the root assay. One gene, *FsPP2C2,* has been identified as a positive regulator of the ABA signal transduction because over-expression of this gene led to enhanced sensitivity of ABA (Reyes *et al.* 2006). However, this is not a likely explanation for the ABA hypersensitivity displayed by the "over-expression" lines in some tissues. More likely is that in these lines the mRNA levels of *SlPP2C1* in some tissues is not higher but lower than in wild type. This would be similar to the co-suppression we observed in the ovary tissue of the over-expressor lines $T6_{OE}$ and $T34_{OE}$. Altogether, the over-expressor lines described here behave a bit extraordinarily and will probably not be useful to study the effect of changed ABA sensitivity in ovary during fruit set. However, the co-suppression lines described above can provide us with the tools to examine the role of ABA in fruit development. Preliminary results indicate that the co-suppression lines carry less fruits on one truss, while the number of flowers per truss is not changed (own observations). ABA-hypersensitive plants thus seem to produce fewer fruits, which would be in agreement with an inhibitory role for ABA during fruit set. Future research will however be necessary to confirm these data and dissect the process further.

In summary, *SlPP2C1* gene seems to be a negative regulator of the ABA response similar to *AtABI1* and *AtABI2*. It is one of few that are described in species other than Arabidopsis. Additionally, the co-suppression lines might shine new lights on the role of ABA in the mature ovary and during fruit set.

Materials and methods

Plant material

Tomato plants (*Solanum lycopersicum* L. cv. Moneymaker from Enza Zaden, Enkhuizen, the Netherlands) were grown under greenhouse conditions from March to October under 16/8h day-night rhythm. Supplementary lights (600 Watt high pressure sodium lights, Philips, Eindhoven The Netherlands) turned on below 200 W/m^2 and turned off above 300 W/m^2. Temperature was kept above 20°C during the light period and 17°C during the dark period with the PRIVA Integro versie 724 system. Plants were watered daily and given fertilizer weekly. Leaf and ovary tissues were dissected form adult tomato plants and root and hypocotyls from 10-day-old seedlings. Tissues were harvested between 11.00 hrs and 13.00 hrs and directly frozen in liquid nitrogen.

Gene-expression analysis

cDNA-AFLP was performed as described in Vriezen *et al.* (2008). For quantitative RT-PCR analysis RNA was isolated with the Trizol (Invitrogen, Carlsbad, CA, USA). Photometric RNA measurements were done to equilibrate the RNA concentrations of different samples. Equal amounts of RNA were DNAse treated with RNAse free DNAse (RQ1, Promega, Madison, USA). RNA (0.5 µg) was reverse transcribed (RT) using a cDNA synthesis kit (iScript™, Bio-rad Laboratories, Hercules, CA, USA) following protocol. Real-time-quantitative RT-PCR (Q-PCR) primers (5'-TCGGAAGGAGAAGATTACG-3' and 5'-TCCACAATTCGCAAC
AAC-3') were designed using a computer program (Beacon Designer Software, Premier Biosoft International, CA, USA), and checked for cross-homology with other PP2C sequences. PCR reactions were performed in a 96-well thermocycler (Bio-Rad iCycler, Bio-rad laboratories) using SYBR green mix (iB-SYBR Green supermix, Bio-rad laboratories). The PCR program started with 3 minutes at 95°C then 40 cycles consisting of 15 second at 95°C and 45 seconds at 57°C and finally the melting temperature of the amplified product was determined to verify the presence of a specific product. Five micro liter of 25-fold diluted cDNA was used per sample. Technical and biological replicates were always performed. Both Actin 2/7 (5'-GGACTCTGGTGATGGTGTTAG-3' and 5'-CCGTTCAGCAGTAGTGGTG-3') and Ubiquitin 7 (5'-CCCTGGCTGATTACAACATTC-3' and 5'-TGGTGTCAG

TGGGTTCAATG-3') were used as internal control genes. Dilluted DNase treated RNA was also included in the Q-PCR as a control for genomic DNA contamination.

Neighbor-joining analysis

ABA-associated PP2C gene sequences of Arabidopsis (described by Schweighofer *et al*. 2004) were obtained from NCBI (www.ncbi.nlm.nih.gov), and used to search for putative PP2C sequences from tomato in the Gene indices database (http://compbio.dfci.harvard.edu/tgi). Eight tomato ESTs were found to have homo-logy on nucleotide or amino acid level to the Arabidopsis genes, listed in table 1.

Table 1: ESTs homologous to ABA related PP2Cs of Arabidopsis

TC nr	name	% homology AA	% homology nt	Length (nt)	*A.thaliana* gene name
TC199352	*SlPP2C1*	42% At2g29380	31% *At2g29380*	1386	
TC201255	*SlPP2C2*	78% At4g26080	60% *At4g26080*	560	*AtABI1**
TC196109	*SlPP2C3*	49% At4g26080	42% *At4g26080*	1460	*AtABI1**
TC197653	*SlPP2C4*	61% At3g11410	54% *At3g11410*	1598	*AtPP2CA*
TC206974	*SlPP2C5*	53% At1g17550	55% *At1g17550*	1869	*AtHAB2*
TC215996	*SlPP2C6*	48% At5g51760	41% *At5g51760*	1438	
TC197648	*SlPP2C7*	57% At3g11410	44% *At2g29380*	1812	AtPP2CA
TC199796	*SlPP2C8*	61% At2g29380	45% *At2g29380*	1413	

* Putative tomato PP2Cs homologous to *AtABI1* are similarly homologous to *AtABI2*

The Arabidopsis and tomato sequences were translated to protein and aligned with ClustalW (www.ebi.ac.uk/clustalw/). A neighbor joining tree was generated using the PHYLIP protdist program (Phylogeny Inference Package version 3.5c; Felsenstein, J. Department of Genetics, University of Washington, Seattle) available on http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py?form=protdist . Default parameters were used, random seed number was 65 (=4n+1) and 100 bootstrap replicates were generated. The consensus tree was drawn in TreeView (free available from taxonomy.zoology.gla.ac.uk /rod/treeview.html).

Plant transformation

To generate transgenic *SlPP2C1*- lines, the coding region of TC199352 (base pair 72-1308 bp) was PCR amplified and cloned in pDONR vector. Using Gateway cloning the *SlPP2C1* was cloned behind the *Cauliflower Mosaic Virus 35S* promoter in the pAD625 vector (de Folter *et al.* 2006) which also contains a *NOPALINE SYNTHASE* terminator. Transgenic plants were generated by *Agrobacterium tumefaciens*-mediated transformation and tissue culture as described in De Jong *et al.* (2008)

Water stress experiment

The pots, of wild type and *SlPP2C1* transgenic lines of the same age and size, were saturated with water at the start of the experiment. Plants were with held form water for ten days. Photographs depicted here were taken nine days after start of the experiment.

Seed germination and root assay

Seeds of the transgenic lines were harvested, dried and stored at 4 degrees. Seeds were sterilized in diluted bleach (4% (w/v) hypochlorite) containing 0.1% (v/v) tween-20, washed and sown on 1/2MS media (2.25 mg MS basal salts supplied with 1% (w/v) sucrose, and Nitsch vitamins, Duchefa, Haarlem, the Netherlands) containing, 0, 1, 3, 10, or 30 μ*M* ABA (Acros, Geel, Belgium). At least 40 seeds per ABA concentration were used. The plates were placed in a growth chamber at 25°C under 16/8h day-night rhythm. Seed germination (radicle protrusion) was scored after ten days and seed germination percentages were calculated.

Seeds were sterilized as described above and sown in water containing 1μ*M* GA_3. After radicle protrusion ten seeds of each line were placed on 1/2MS media containing either 0, 5, 10, or 40 μ*M* ABA. The roots were measured and the plates were placed in vertical position and grown under conditions described above. Seven days after transfer, the length of the root was measured again and root growth was calculated as a percentage of root growth compared to 1/2MS media without ABA (100%). Experiments were carried out three times and mean values plus SD are shown. Student's t-tests were performed to test for significance ($p<0.05$).

Chapter 6

SlDRM1, a dormancy gene, is strongly regulated by pollination, auxin and gibberellin through its 3´-UTR

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Abstract

Dormancy, the temporary inhibition of growth, occurs in seeds, axillary buds and apical buds. The *DRM*-genes have been identified as good dormancy markers in pea and we have recently identified a homologue of these genes in tomato. This gene, designated *SlDRM1*, is expressed at low levels during ovary development but mRNA levels strongly increase at ovary maturity. Pollination, auxin and gibberellin treatments reduce the mRNA levels of *SlDRM1* and this is mediated by the 3´-UTR, suggesting that transcript stability is altered by these treatments. The mRNA is mainly localized in the ovules, more specifically in the cells surrounding the embryo sack, suggesting that here *SlDRM1* may have a function.

Introduction

Dormancy is a widely used, though somewhat ambiguous, concept describing the temporary arrest of growth which is often associated with unfavorable environmental circumstances. Efforts made to understand the mechanism of dormancy are complicated by the fact that there are several different forms of dormancy, which might simultaneously control the inhibition of growth (Horvath *et al.* 2003). Three main forms were defined by Lang *et al.* (1987): paradormancy, when the inhibition of growth is caused by distal organs; endodormancy, when the inhibition of growth is caused by internal signals; ecodormancy, when the inhibition of growth is caused by temporary unfavorable environmental conditions. There are many examples of dormancy within the life cycle of a plant, most well known are seed dormancy, axillary bud dormancy, and apical bud dormancy. Dormancy is regulated by a complex network of different hormones interacting with each other, while environmental factors may further complicate this regulatory network. In general, abscisic acid (ABA) is important for dormancy induction and maintenance, while the balance between ABA and stimulating substances (e.g. gibberellins and auxin), determines the release from dormancy (Finkelstein *et al.* 2008; Cline and Oh 2006; Ruttink *et al.* 2007).

Before pollination and fertilization occur, the mature ovary is also temporarily halted in its growth and development. Gene expression data indicate that the mRNA levels of cell cycle genes, for instance *CDKA1, CDKB1* and *CDKB2*, and *CycD3,* are relatively low in mature ovaries. By contrast, the mRNA levels of dormancy markers such as *DRM1* and *DRM3* are relatively high (Vriezen *et al.* 2008). Therefore gene expression patterns in the mature ovary before pollination suggest that it is in a dormancy-like state. Growth and

development of the ovary after pollination is induced by auxin and gibberellin (Gillaspy *et al.*1993). Furthermore, there are indications that ABA may be an additional inhibitory player in this regulatory network (Vriezen *et al.* 2008; chapter 2), comparable to dormancy in other tissues.

As mentioned before *DRM*-genes have been described as dormancy markers. They encode proteins that are annotated as dormancy associated and auxin repressed (DRM/ARP). Members of the *DRM/ARP*-gene family have been identified in several species such as tobacco, strawberry and pea (Reddy and Poovaiah 1990; Stafstrom *et al.* 1998; Steiner *et al.* 2003). In Arabidopsis five gene-loci are annotated as *DRM/ARP* genes (At1g56220, At1g54070, At2g3380, At1g28330, and At5g44300). Also in pea three *DRM/ARP*-genes were described (AF029242, AF515795 and AF515796), suggesting that they occur in small gene families within one species. Expression studies have linked the expression of these genes to arrested growth. For instance, the mRNA levels of *PsDRM1*, a homologue in pea, were always high in dormant buds and decreased after activation of growth. *PsDRM1* mRNA levels were also high in mature roots or fully elongated stems that had arrested growth (Stafstrom *et al.* 1998). Additionally, the mRNA level of *RpARP1,* a *DRM/ARP*-gene in *Robinia pseudoacacia*, was higher in segments of shoots that grew slowly, and lower in segments that grew fast (Park and Han, 2003). In *Nicotiana tabacum* the *Arpl1-1* gene was associated with pollen maturation and its mRNA levels were strongly reduced at pollen germination (Steiner *et al.* 2003). A function for the *DRM*-genes has not been reported. The *DRM/ARP* genes are also annotated as auxin repressed genes and this has been shown in fruits of strawberry and hypocotyls of black locust, in which transcript levels decreased after auxin treatment (Reddy and Poovaiah 1990; Park and Han 2003). Interestingly, if the *RpARP1*gene was expressed in Arabidopsis without its own 5`UTR and 3`UTR sequences auxin did not affect mRNA levels any more. In contrast, auxin did reduce mRNA levels if the UTR sequences were included in the transgene. Park and Han (2003) therefore suggested that the gene expression was post-transcriptionally regulated via its UTRs. We previously identified a homologue of these *DRM*-genes in tomato, which has been designated *SlDRM1*. Since there are so many parallels between dormancy processes and the status of the mature ovary before pollination, studies of the expression and regulation of the *SlDRM1* gene during ovary and fruit development could shed a light on the dormancy-like conditions of the mature ovary.

Results

Isolation of the *SlDRM1* gene and promoter

SlDRM1 has been identified by us from a transcriptome analysis. Its mRNA level was high in unpollinated ovaries and low after fruit set (Vriezen *et al.* 2008). The full length mRNA sequence was obtained with a PCR based approach using a cDNA library of tomato ovaries as a template (see materials and methods). The *SlDRM1* transcript is 634 nucleotides (nt) long with a 26 nt 5`-UTR and a 238 nt long 3`-UTR. It codes for a 123 amino acid long protein with a calculated molecular weight of 13.4 KDa. A Kyte-Doolittle hydrophobicity-plot revealed that only the C-terminal first six amino acids are hydrophobic, while the rest of the sequence is hydrophilic. The protein sequence shares more than 90% identity on amino acid level with putative proteins encoded by auxin repressed (ARP)-dormancy associated (DRM) genes identified in the Solanaceae family (*Nicotiana tabacum*, *Capsicum annuum* and *Solanum virginianum*), and at lower levels also with *ARP/DRM*-genes from other plant species, such as *Arabidopsis thaliana*, *Pisum sativum*, and *Robinia pseudoacacia*. An alignment of the products of *ARP/DRM* genes from several species identified highest homology in the C-terminal region (Fig. 1A). The phylogentic neighbor-joining analysis identified two clades of which one is only comprized of sequences from the Solanaceae family (Fig. 1B). We performed a genome-walk experiment and obtained a putative promoter region of 1412 base pairs (bp) of the *SlDRM1* gene. A scan for *cis*-acting-regulatory-elements (CARE) of the promoter region revealed the TATA-box at -48 bp before the transcription start (0), a putative ethylene response element (ERE) at -355 bp, and a putative GT1-light responsive element at -460 bp (Fig. 2).

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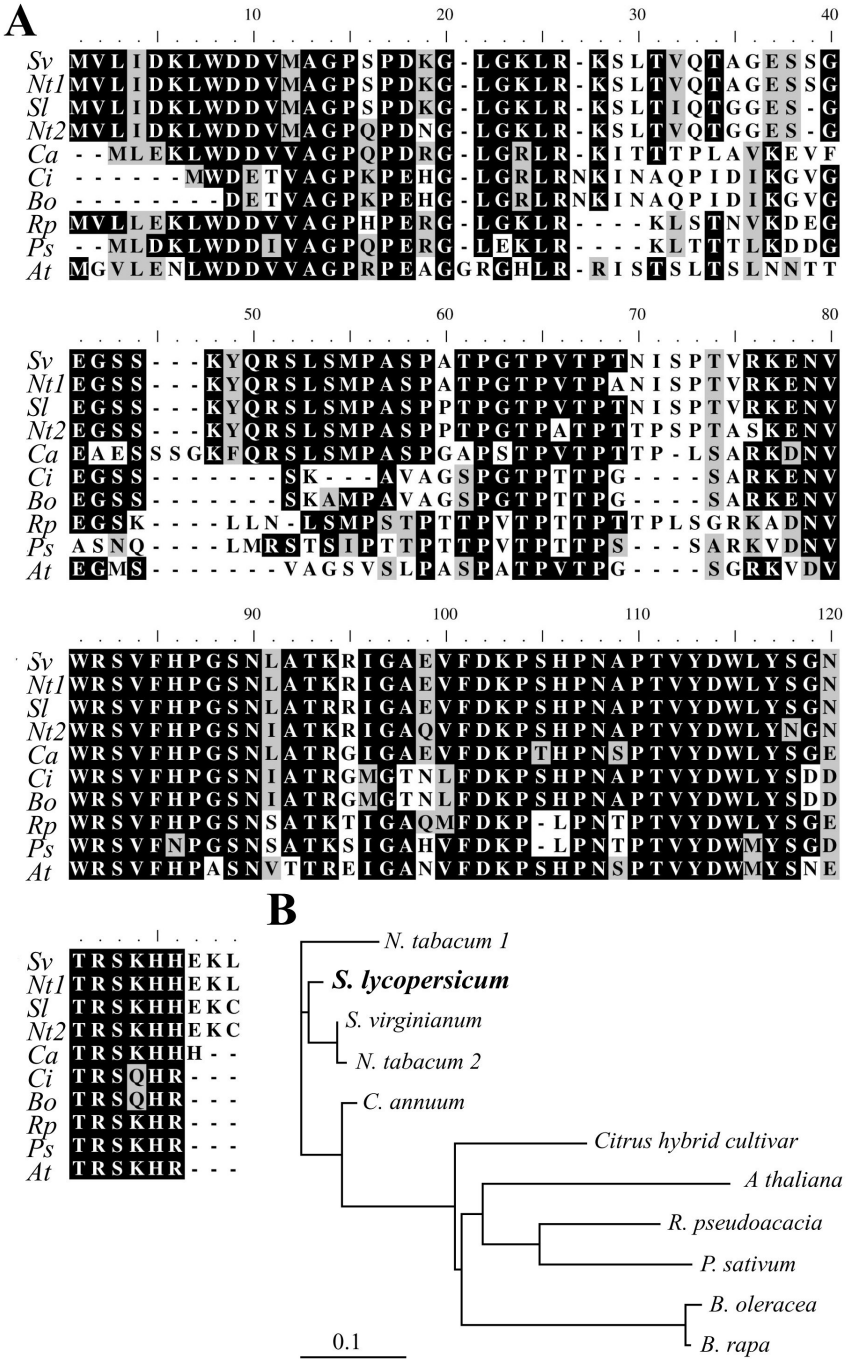

Fig. 1: A) Alignment of DRM/ARP amino acid sequences of several species. Overall homology is quite high, but the C-terminal part of the sequence is homologous in all species. Abbreviations in alignment are first letter of genus and species name, except citrus hybrid cultivar (Ci). **B)** The neighbor-joining tree shows that the sequences of species of the Solanaceae family (*N. tabacum*, *S. virginianum*, *C. annuum*, and *S. lycopersicum*) form a clade. Identical amino acids are depicted in black shading and similar amino acid in grey shading. For accession details see materials and methods.

Fig. 2: Partial promoter sequence of *SlDRM1* showing TATA-box (underlined) at -48 bp, ethylene responsive element (ERE, black box) at –355 bp and the GTI-light responsive element (grey box) at –450 bp.

SlDRM1 expression in flower and early fruit development

We performed a quantitative real time PCR (Q-PCR) experiment to analyze the expression of *SlDRM1* in the ovary throughout flower and early fruit development (Fig. 3). *SlDRM1* mRNA levels were relatively low in the ovary during flower development, but strongly increased at the day of anthesis. From 48 hours after pollination onwards mRNA levels were low again.

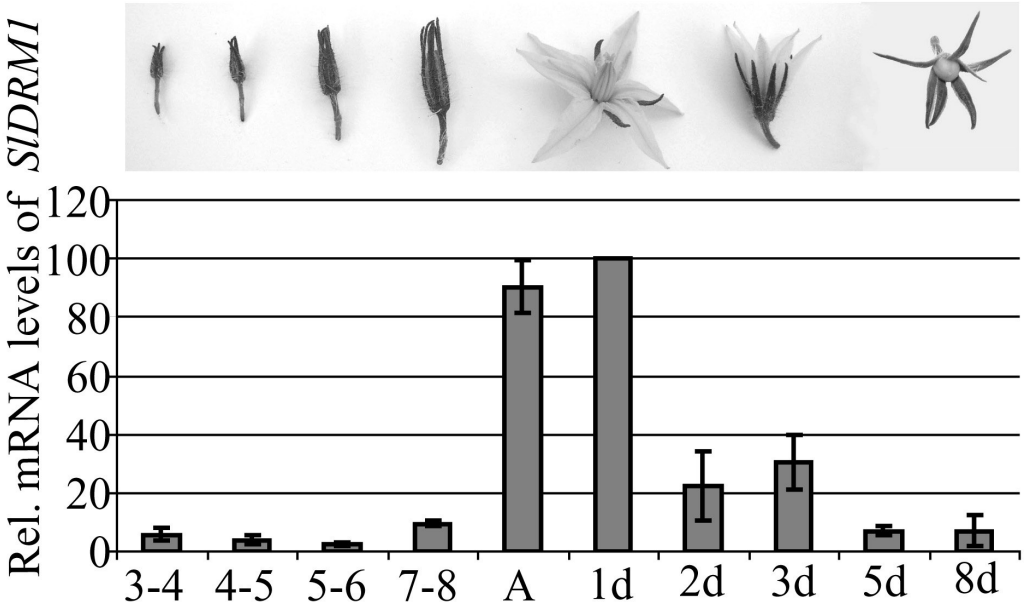

Fig. 3: Relative mRNA levels of *SlDRM1* increase at anthesis and decrease again two days after pollination. In young fruits the levels are low and comparable to the levels in ovaries during development. Biological replicas and SE are shown. RNA was isolated from ovaries of 3-4 mm buds (3-4), 5-6mm buds (5-6), 7-8 mm buds (7-8), unpollinated flowers at anthesis (A) and 1, 2, 3, 5, 8 days after pollination (1dap, 2dap, 3dap, 5dap, 8dap). The flower stages are depicted in the picture above the graph.

In situ hybridization localized the mRNA of *SlDRM1*in the ovules and outer placental layers of unpollinated ovaries (Fig. 4A). The purple staining in ovaries three days after pollination was less strong compared to unpollinated ovaries. The sense-probe gave no staining. Within the ovule the strongest purple staining was observed in the cell layers around the embryo-sack. Quantitative PCR confirmed the localization of *SlDRM1* mainly in the ovules and placental tissue (Fig. 4B). In all three tissues a significant reduction in mRNA levels of *SlDRM1* after pollination was observed. Altogether this indicates a tight control of the *SlDRM1* expression in ovary. To examine this better we expressed the gene encoding for enhanced yellow fluorescent protein (eYFP) under control of the *SlDRM1* promoter in combination with its 5`- and 3`-UTR sequences. In transgenic plants expressing this construct, *eYFP* mRNA levels were reduced three days after pollination, comparably to *SlDRM1* mRNA levels (Fig. 5B). We observed strong yellow fluorescence in the ovules and outer placental tissue in unpollinated ovaries (Fig. 4C). The strongest fluorescence was observed in the cell

layers surrounding the embryo-sack. Some yellow fluorescence was also observed in the pericarp. Three days after pollination fluorescence was still observed in the ovules.

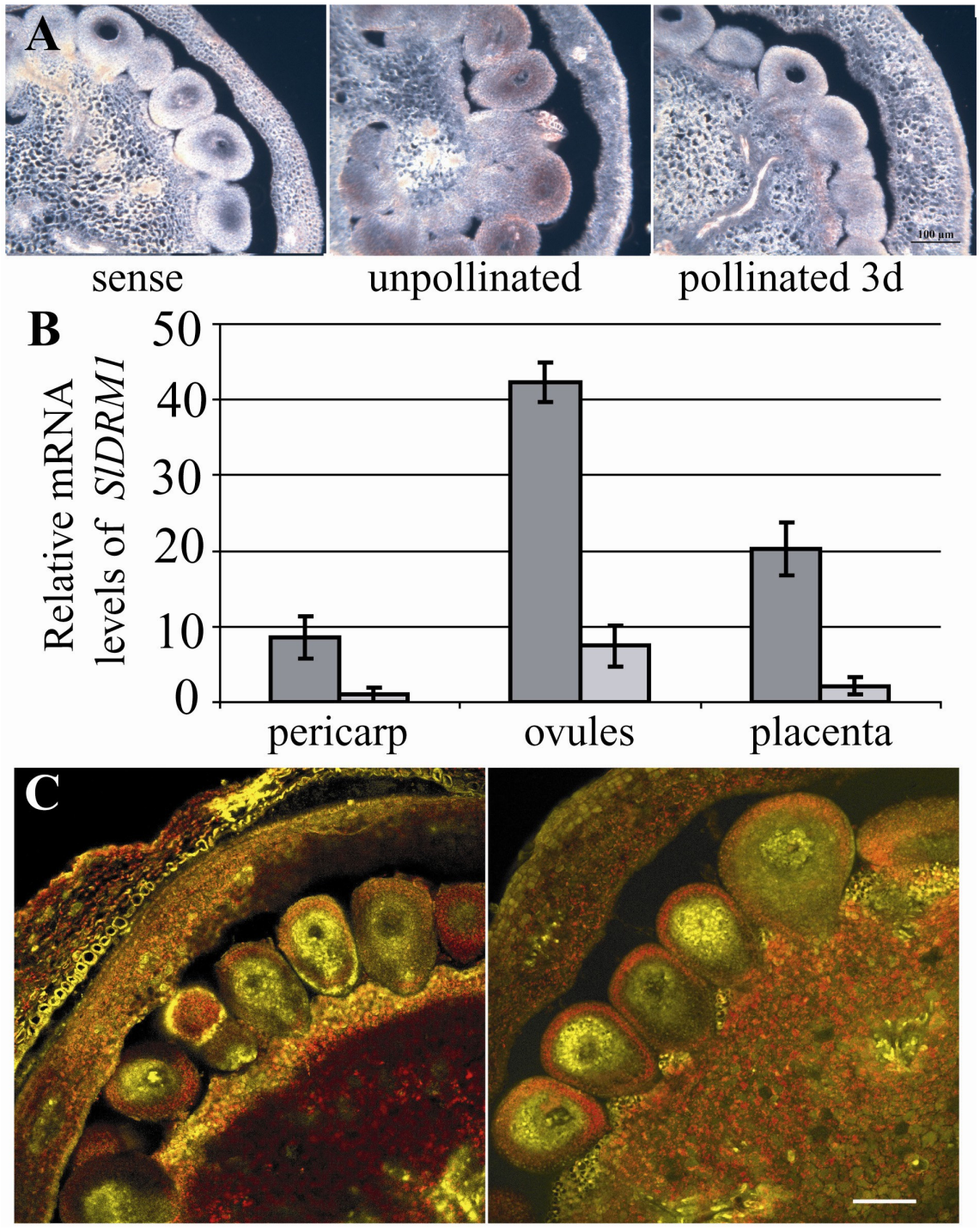

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Fig. 4: A) *in-situ* hybridization with *SlDRM1* shows that the gene is most abundantly expressed in the ovules, especially the cells surrounding the embryo-sack. Hybridization with a sense probe (control, left), anti-sense-probe in unpollinated ovaries (middle) and in ovaries three days after pollination (right) are depicted, scale-bar represents 100µm. **B)** Quantitative PCR in the different tissues of the ovary before pollination (dark grey bars) and three days after pollination (light grey bars). mRNA levels of *SlDRM1* are highest in unpollinated ovules. After pollination in all three tissues there is a decrease in *SlDRM1* mRNA levels. Mean values of biological replicas and SE are shown. **C)** eYFP-fluorescence (yellow) observed in the unpollinated (left) and pollinated (right) ovaries of a tomato plant expressing the *eYFP*-reporter gene under control of the *SlDRM1* promoter and its UTR-sequences. Fluorescence is observed mainly in the ovules, specifically in the cells surrounding the embryo-sack, and slightly in the outer placental cell layers and pericarp. In the ovaries three days after pollination fluorescence is observed in the ovules. Auto-fluorescence (red) in general is not co-localized with eYFP fluorescence, and scale bar represents 100µm.

Hormonal regulation of *SlDRM1*

The *SlDRM1* gene activity seems regulated by different hormones. In general, *DRM* genes are annotated as auxin-repressed genes. Additionally, *RpARP1* and also *SlDRM1* mRNA levels are also affected by GA_3-application (Park and Han 2003; Vriezen *et al.* 2008). Therefore we set to study the hormonal regulation of *SlDRM1* in more detail (Fig. 5A). Interestingly, mRNA levels of *SlDRM1* were still high three days after anthesis if the flowers were not pollinated (control, C). As soon as pollination occurred, mRNA levels decreased considerably. Also GA_3 and IAA treatment of unpollinated ovaries reduced the mRNA level of *SlDRM1*. ABA-treatment did not influence the *SlDRM1* expression significantly and showed considerable variation between biological replicas.

Park and Han (2003) suggested that the UTR-sequences might be important for the auxin repression of the *RpARP1* gene. *ARP/DRM* genes have a relatively large 3`UTR sequence. The *SlDRM1* mRNA contains a 238 nt 3`UTR which seems relatively conserved in the *DRM* genes of other species; it shares 72% homology with the whole DRM 3`UTRs of *N. tabacum* (Genbank accession number AY572223) *S. virginianum* (AY572222) and 84% with that of *S. tuberosum* (Dane Farber Cancer Institute Tentative Consensus TC167371). Therefore it is plausible that the 3`UTR plays a role in the regulation of the mRNA levels of the *SlDRM1* in the ovary. To determine if such a regulatory mechanism plays a role, we

constructed three transgenes containing the *eYFP*-coding region under control of the *SlDRM1*-regulatory sequences (promoter, 5`UTR and 3`UTR) or under control of the *Cauliflower Mosaic Virus promoter 35S*-promoter (*p35S*), followed by either the *SlDRM1* 3'UTR (*DRM1*) or the *NOPALINE SYNTHASE* 3'UTR (*NOS*). Plants expressing the *pSlDRM1*::*eYFP*::*DRM* construct had *eYFP* mRNA levels comparable to that of the endogenous *SlDRM1* gene, either in the wild type (Fig. 5A) or in transgenic plants (Fig. 5B). This suggests that all regulatory elements are present in the construct. In addition, CLSM analysis localized the eYFP signal in the same tissues as the *in situ* RNA hybridization did for the endogenous *SlDRM1* mRNA (Fig. 4C). Substitution of the *SlDRM1* promoter for the *CaMV35S* promoter (*p35S*::*eYFP*::*DRM*) did not change the pattern of *eYFP* expression. Auxin or gibberellin applications still led to a decrease of the *eYFP* mRNA (Fig. 5C). Expression of the *eYFP* gene under control of the *CaMV35S*-promoter and the *NOS*-UTR (*p35S*::*eYFP*::*NOS*) was not significantly altered as compared to control by any of the treatments (Fig. 5D). Endogenous *SlDRM1* gene expression was used in every eYFP-experiment as a control for the various treatments.

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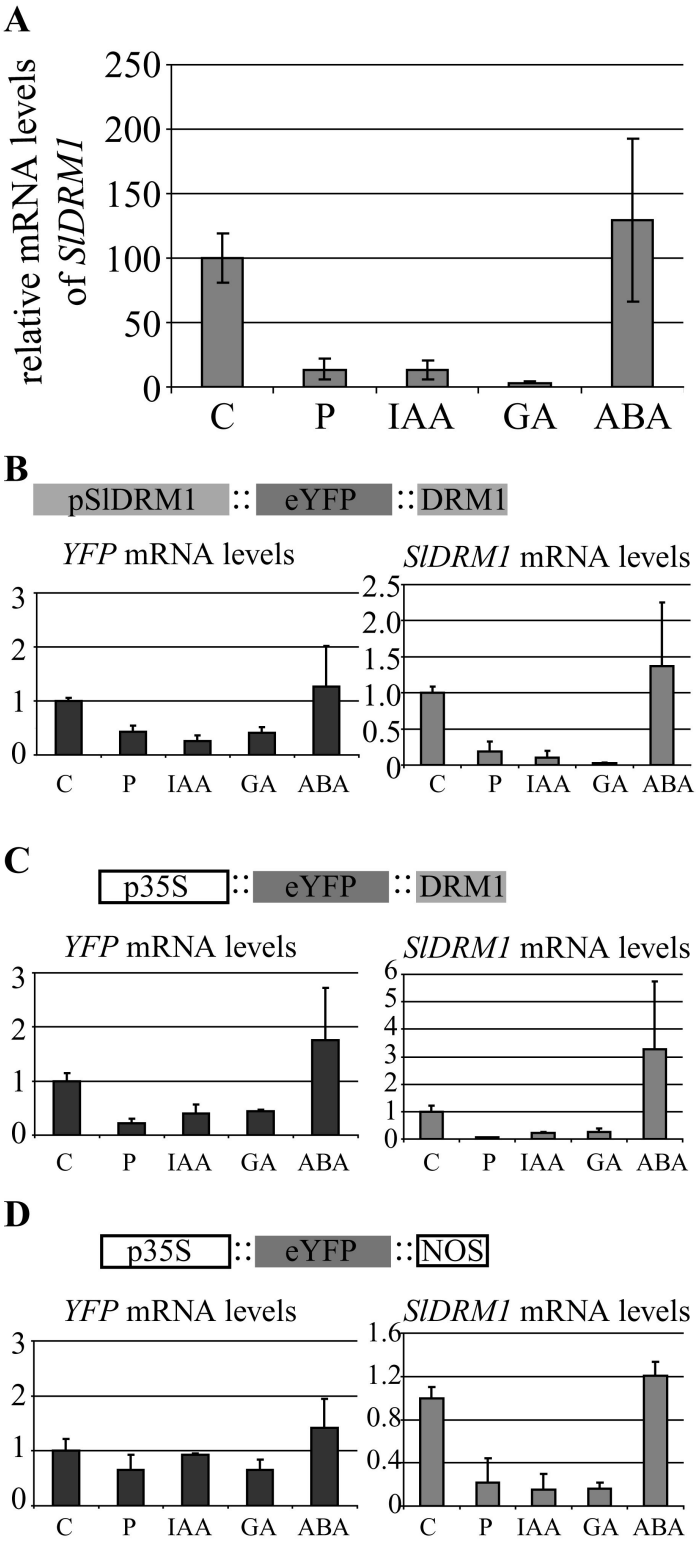
Fig. 5: A) Relative mRNA levels of *SlDRM1* are reduced three days after pollination (P), or after treatment with IAA or GA (IAA, GA). No significant influence of ABA treatment was detected. **B)** Relative mRNA levels of *eYFP* expressed under control of the *SlDRM1* promoter and UTRs are similar to the endogenous *SlDRM1* mRNA levels. **C)** Also the mRNA levels of *eYFP* expressed under control of the *CaMV35S* promoter and 3` UTR of *SlDRM1* are similar to endogenous *SlDRM1* mRNA levels. **D)** Relative mRNA levels of *eYFP* expressed under control of *CaMV35S* promoter and *NOPALINE SYNTHASE* 3` UTR are not influenced by any of the treatments. Endogenous *SlDRM1* expression in B, C, and D is always similar to expression in wild type A. Mean values of biological replicas and SE are shown.


A
relative mRNA levels of SlDRM1
250
200
150
100
50
0
C
P
IAA
GA
ABA
B
pSlDRM1 :: eYFP :: DRM1
YFP mRNA levels
SlDRM1 mRNA levels
C
p35S :: eYFP :: DRM1
YFP mRNA levels
SlDRM1 mRNA levels
D
p35S :: eYFP :: NOS
YFP mRNA levels
SlDRM1 mRNA levels

Discussion

The *SlDRM1* gene is homologous to dormancy associated/auxin-repressed genes (*DRM/ARP*-genes) from several species. The sequence identity on amino acid level between family members of different species is high; ranging from 60% between tomato and Arabidopsis members till 95% between tomato and tobacco members. The strongest conservation can be found in the C-terminal part of the proteins, indicating that this region is probably functionally very important. In addition to the distinct conservation on the protein level, also the intron-exon structures of the *DRM/ARP*-genes are highly conserved. We found that the genomic sequence of *SlDRM1* (DQ672601; determined by Manning *et al.* 2006) has the same intron-exon structure as the five Arabidopsis family members, which means that the structures of the genes have been conserved over more than 120 Million years of evolution. Altogether this suggests that the genes have an important function in the life cycle of plants, as has also been mentioned by Park and Han (2003).

Despite the interesting features mentioned above, no functions for DRM-proteins have been elucidated yet. Over-expressing *DRM/ARP*-genes (*RpARP1* and *EuNOD-ARP1*) in Arabidopsis did not lead to any aberrant phenotypes (Park and Han 2003; Kim *et al.* 2007). Neither are there any reports of modified phenotypes in knock-down or knock-out lines. Moreover, we could also not yet observe any aberrant phenotype in transgenic tomato plants expressing a RNAi-construct or over-expression-construct under control of the CaMV 35S-promoter (data not shown). Indications for the function of *SlDRM1* in ovaries might thus be sought in the localization of its mRNA, which is mainly found in the ovules, more specifically in the cells surrounding the embryo-sack. Together with the knowledge that high mRNA levels are present in unpollinated ovaries at anthesis, this might suggest that *SlDRM1* is important for the fertilization process or for the embryo-sack at maturity. However, a very specific function in ovules is not very likely since *DRM*-genes are expressed in several growth arrested tissues other than the ovary (Kim *et al.* 2007; Park and Han, 2003; Stafstrom *et al.* 1998; Steiner *et al.* 2003). Therefore a more general function is to be expected, perhaps influencing the cell cycle machinery. In many dormancy processes the growth arrest is related to survival of the plant under unfavorable environmental conditions, and protective mechanisms are induced concomitant with dormancy induction (Finkelstein *et al.* 2008; Ruttink *et al.* 2007). Interestingly, in hot pepper a putative *DRM/ARP* gene was isolated that was higher expressed under cold and salt stress, and *RpARP1* was also induced by cold stress (Park and Han 2003; Hwang *et al.* 2005). Thus, another putative function for the *SlDRM1*

gene in ovaries could be related to the protection of the embryo sack at maturity. The function of *SlDRM1* might be redundant with the gene product of other *DRM*-genes. Expression of DRM-genes in fruits was reported before (Reddy and Poovaiah 1990). They found that in strawberry fruits *DRM/ARP* mRNA levels decreased after pollination or auxin treatment, which is in agreement with our findings. However, they could not differentiate between the different tissue types of the unpollinated fruit and one should bear in mind that the fruit of strawberry is an aggregate-fruit which is formed also by the receptacle.

Interestingly, the localization of *SlDRM1* in tomato ovaries is comparable to the localization of the hormone ABA in mature unpollinated ovaries of Arabidopsis and cucumber (Peng *et al.* 2006). Additionally, there seems to be a tight correlation between ABA and dormancy induction (Finkelstein *et al.* 2008; Cline and Oh 2006; Ruttink *et al.* 2007). Since *SlDRM1* is induced at ovary maturity when growth is arrested and ABA levels are high (chapter two), this might suggest that ABA regulates *SlDRM1* gene-expression. However, we found that ABA-treatment of mature ovaries did not change *SlDRM1* expression significantly, suggesting there is no transcriptional regulation of *SlDRM1* by ABA. ABA treatment did also not influence *RpARP1* mRNA levels (Park and Han 2003). The strong variation in *SlDRM1* expression between biological replicas treated with ABA might be related to differences in uptake of ABA by the ovary when the hormone is exogenously applied. Other ABA-responsive genes however, were always found to be induced in the same RNA/cDNA preparations (own observation; chapter 2). Possibly, the interaction between *SlDRM1* and ABA takes place at the level of translation. Therefore it would be interesting to quantify SlDRM1 protein levels in wild type plants or eYFP protein levels in our *eYFP*- reporter-lines. Especially, as there seems to be a functional link between RNA metabolism and ABA signaling which in turn can influence translation efficiency (Kuhn and Schroeder 2003; Wasilewska *et al.* 2008). The *SlDRM1* transcript level decreased after auxin and gibberellin treatments. This also happened with the transcript of a reporter gene which was fused to the *SlDRM1* 3`UTR, suggesting that the hormonal regulation is mediated by this trailer sequence. The 3`UTR is known to be important for transcript stability, its localization and translation initiation (Grzybowska *et al.* 2001). Thus, it is conceivable that auxin and gibberellin decrease the stability of the *SlDRM1* transcript. Most *DRM*-genes are annotated to be auxin repressed, and the gene-expression of *RpARP1*, a *DRM*-gene in *Robinia psuedoacacia*, was most likely also post-transcriptionally reduced by auxin via its UTRs (Park and Han 2003). Therefore it would be interesting to look for motifs that are related to auxin and possibly also gibberellin

induced transcript instability within the different 3`UTR sequences of several species. However, if the full-length cDNA (including UTRs) of an *Elaeagnus umbellata DRM/ARP* gene was expressed under a *Cauliflower Mosaic Virus 35S*-promoter auxin did not change expression levels (Kim *et al.* 2007). The authors therefore suggested that altering the stability of *DRM/ARP* mRNA is not a general mechanism for controlling DRM gene-expression, but in *Robinia pseudoaccacia* and tomato it most likely is. *SlDRM1* mRNA levels decrease after pollination most likely because auxin and gibberellin levels increase (Gillaspy *et al.* 1993). In the promoter we found an ethylene responsive element, suggesting that also this hormone may regulate the *SlDRM1* transcript levels. Indeed we found that 1-MCP treatment reduced *SlDRM1* mRNA levels (data not shown). Notably, gene expression of ethylene biosynthesis genes and ethylene response genes, such as ACOs, ERFs and EREBPs, seems to be relatively high before pollination and decrease afterwards, suggesting that also ethylene levels are high in unpollinated ovaries (Vriezen *et al.* 2008). Possibly, induction of the *SlDRM1* transcript in ovaries at anthesis is regulated by ethylene. Altogether the function of *DRM/ARP* genes remains very intriguing because of the high amino-acid sequence conservation between species, the cell specific localization of the mRNA and the complex and extensive regulation of their expression.

Acknowledgements

We like to thank Dr. M. Bemer (Radboud University, Nijmegen) for very useful scientific discussions on gene conservation and evolution. We are also grateful to Dr. E. Pierson (General Instrumentarium, Radboud University, Nijmegen) for assistance with CLSM.

Materials and Methods

Plant material and hormone treatments

Tomato plants (*Solanum lycopersicum* L. cv. Moneymaker from Enza Zaden, Enkhuizen, the Netherlands) were grown under greenhouse conditions from March to October under 16/8h day-night rhythm. Supplementary lights (600 Watt high pressure sodium lights) turned on below 200 W/m^2 and turned off above 300 W/m^2. Temperature was kept above

20°C during the light period and 17°C during the dark period with the PRIVA Integro versie 724 system. Plants were watered daily and given fertilizer weekly. Before hormone treatment flowers were emasculated 3 days before full bloom (dbf) and at full bloom either hand pollinated or treated with 2 µL of either 1 m*M* GA_3 (Duchefa, Haarlem, the Netherlands) in 1% (v/v) ethanol, or 1 m*M* 4-Cl-IAA (Sigma-Aldrich, St. Louis, MO, USA) in 2% (v/v) ethanol, or 1 m*M* ABA (Acros, Geel, Belgium) in 10% (v/v) methanol. 4-Cl-IAA is a more stable form of auxin than IAA and was used because it appeared to induce tomato fruit set more reliably than IAA. Control flowers were treated with 2µL of the solvent (10% methanol or 1% ethanol). Hormone treatments were repeated after two days. Flowers were harvested three days after first treatment unless otherwise noted. Plant material was sampled between 11.00h and 13.00h and directly frozen in liquid nitrogen. When necessary, plant material was dissected using binoculars and frozen thereafter in liquid nitrogen. The leaf and ovary tissues were dissected from adult plants. Roots and hypocotyls were isolated from 10-day-old seedlings.

Full length cDNA and promoter isolation

The full length *SlDRM1* sequence was isolated by PCR on a phage cDNA library (HybriZAP® 2.1, Stratagene, La Jolla, CA, USA) with primers based on the adapter sequence (5'- TCTATTCGATGATGAAGATACCCCACC-3' and 5'- CTGCAGTAATACGACTCAC TATAGGGC-3') and gene-specific primers for *SlDRM1*(5'- GAGCCTCACTATTCAAACT G-3' and 5'- AAACACTCCTCCACACATTC-3'), which were based on the available sequence information of the cDNA-AFLP fragment. The PCR was performed using one micro liter of 10-fold diluted cDNA-library, buffer IV, 3.5 m*M* $MgCl_2$, 0.5unit redhot Taq DNA polymerase (all from ABgene limited, Epsors, UK), 0.4 mM dNTPs (Fermentas, St Leon-Rot, Germany), and 0.1 µ*M* primers in a PCR with 6 minutes at 96°C followed by 30 cycles each comprising of 30 seconds at 95°C, 30 seconds at 60°C and 2 minute at 72°C. The promoter sequence was obtained via a genome walk experiment (Genome-Walker universal kit, BD Biosciences, CA, USA) on EcoRV, Eco72I/EcoRV and SnaI (Fermentas) GenomeWalker-libraries using the nested gene-specific primers (5'-ATGTCAAAAAGCGC GGATCATCAA TTATCA-3' and 5'-GCGCGGATCATCAATTATCACTGAAGAAG-3').

Bio-informatics and sequence-alignment

The molecular weight of *SlDRM1* was calculated with the freely available web based tool at http://www.encorbio.com/protocols/Prot-MW.htm (Encore Biotechnology *inc*.). A kyte-Doolittle hydrophobicity-plot was made with the freely available web based tool at http://www.vivo.colostate.edu/molkit/hydropathy/. Auxin-repressed dormancy-associated gene sequences from other species were found based on homology with *SlDRM1* in a discontinuous tblastX at NCBI (www.ncbi.nlm.nih.gov) or from literature. The gene-sequences were translated to protein and aligned with ClustalW (www.ebi.ac.uk/clustalw/). A prodist neighbor-joining tree was generated using BioEdit available at http://www.mbio.ncsu.edu/BioEdit/BioEdit.html (Ibis Biosciences, Carlsbad CA, USA). The *cis*-acting regulatory elements (CARE) scan was perform via a web based tool http://bioinformatics.psb.ugent.be/webtools/plantcare/html/ (freely available).

Gene-expression

For quantitative PCR analysis RNA was isolated with Trizol (Invitrogen, Carlsbad, CA, USA). Photometric RNA measurements were done to equilibrate the RNA concentrations of different samples. Equal amounts of RNA were DNAse treated with RNAse free DNAse (RQ1, Promega, Madison, USA). RNA (0.5 µg) was reverse transcribed (RT) using a cDNA synthesis kit (iScript^{tm}, Bio-rad Laboratories, Hercules, CA, USA) following protocol. Real-time-quantitative PCR (Q-PCR) primers for *SlDRM1* (5'-AGAGCCTCAC TATTCAAACTG-3' and 5'-AAACACTCCTCCACACATTC-3') and *eYFP* (.5'-AGCAGA AGAACGGCATCAAGG-3' and 5'-CCAGCAGGACCATGTGATCG-3') were designed using a computer program (Beacon Designer Software, Premier Biosoft International, CA, USA). PCR reactions were performed in a 96-well thermocycler (Bio-Rad iCycler, Bio-rad laboratories) using SYBR green mix (iB-SYBR Green supermix, Bio-rad laboratories, Hercules, CA, USA). The PCR program started with 3 minutes at 95°C then 40 cycles consisting of 15 second at 95°C and 45 seconds at 57°C and finally the melting temperature of the amplified product was determined to verify the presence of a specific product. Five micro liter of 25-fold diluted cDNA was used per sample. Technical and biological replicates were always performed. Both Actin 2/7 (5'-GGACTCTGGTGATGGTGTTAG-3' and 5'-CCGTTCAGCAGTAGTGGTG-3') and Ubiquitin 7 (5'-CCCTGGCTGATTACAACATTC-3' and 5'-TGGTGTCAGTGGGTTCAATG-3') were used as internal control genes. Dilluted

DNase treated RNA was also included in the Q-PCR as a control for genomic DNA contamination.

In situ hybridization

The RNA probes were synthesized by transcribing base pairs 389 -640 of the *SlDRM1* gene using T7 RNA polymerase for both sense and anti-sense and digoxigenin-labeled UTP (Roche applied science, Basel, Switzerland). DIG labeling efficiencies were tested by a spot assay. Tissue fixation and hybridization was performed as described in chapter 2.

Plant transformation

To generate reporter lines the promoter (including the 5`UTR) and 3`UTR of *SlDRM1*, the *Cauliflower mosaic virus (CaMV) 35S*-promoter, *NOPALINE SYNTHASE (NOS)*-terminator and enhanced yellow fluorescent protein (eYFP)-reporter gene were all PCR amplified with specific primers that were elongated with the sequence of the gene that needed to be attached to it, for primer sequences see table 1. Afterwards, an assembly PCR was performed on the 100-fold diluted PCR products (1 µL each) with buffer HF, 0.4 U phusion (both from Finnzymes, Espoo, Finland), 0.2 mM dNTPs (Fermentas), and 0.1 µ*M* primers, in a 35 cycle reaction consisting of 10 seconds 98°C, 20 seconds 60°C and 2 minutes 72°C. The assembled PCR products were, sequenced, digested with EcoRI and XbaI (fermentas) and cloned into EcoRI and XbaI cloning sites of the binary pCAMBIA 1300 vector (CAMBIA www.cambia.org, Australia). Transgenic plants were generated by *Agrobacterium tumefaciens*-mediated transformation and tissue culture as described in De Jong *et al.* (2008).

Table 1: primer sequences for assembly PCR of reporter lines

name	Primer-sequence	part
DRMp-Fwd	CTGAATTCATATGACTTATCTTCTTCTTAATC	promoter
DRMp-R-eYFP	CTCCTCGCCCTTGCTCACCAT**TTTCTCTCTCTTAGCTTACTATACTA**	promoter
35S-Fwd	GTGTGAATTCCATGGAGTCAAAGATTCAAATAGAGG	promoter

35S-R-eYFP	CTCCTCGCCCTTGCTCACCA**TGGTCAAGAGTCCCCCGTGTTCTC**	promoter
EYFP-F-DRMp	TAGTATAGTAAGCTAAGAGAGAGAAA**ATGGTGAGCAAGGGCGAGGAG**	reporter
EYFP-R-DRM	CCCAAAAACTCGGTACCACA**TCACTTGTACAGCTCGTCC**	reporter
EYFP-F-35S	GAGAACACGGGGGACTCTTGACC**ATGGTGAGCAAGGGCGAGGAG**	reporter
EYFP-R-NOS	CTTTATTGCCAAATGTTTGAACG**TCACTTGTACAGCTCGTCC**	reporter
DRM-R-XbaI	ACACTCTAGAGTGTAAGCTTAGTAATAATAGTAAC	3'UTR
DRM-F-EYFP	GGACGAGCTGTACAAGTGA**TGTGGTACCGAGTTTTTGGG**	3'UTR
NOS-R-XbaI	ACACTCTAGACCCGATCTAGTAACATAGATGACACC	3'UTR
NOS-F-EYFP	GGACGAGCTGTACAAGTGA**CGTTCAAACATTTGGCAATAAAG**	3'UTR

Confocal Laser Scanning Microscopy (CLSM)

YFP-analysis was performed using Confocal Laser Scanning Microscopy (CLSM, Leica TCS-Spa AOBS microscope). Fibrotome sections (100 µm) were made from ovaries three days after pollination. Sections were collected in distilled water and placed on a microscope slide. YFP was excited with an Argon laser (514 nm) and eYFP specific fluorecscent emission was detected between 525 nm and 578 nm (depicted in yellow). Hardware settings were kept similar between different sampels, wildtype sampels were also included but they never gave a significant signal. A control for autofluorescence was made by detection of emission between 612nm and 690nm (depicted in red). CLSM was performed at the general instrumentarium of the science faculty (GI, Radboud university Nijmegen)

Accession details

Genbank nr AA-alignment: *Arabidopsis thaliana* DRM1 (NM102599), *Brassica oleracea* (AF458410), *Brassica rapa* (AY185352), *Capsicum annuum* (AY488487), *Citrus* variety '*Shiranuhi*' (EF122398), *Nicotiana tabacum* 1 (AY572223) *Nicotiana tabacum* 2 (EU286949), *Pisum sativum* (AF029242), *Robinia pseudoacacia* (AY009094), *Solanum virginianum* (AY572222)

Chapter 7

Concluding remarks and perspectives

L.Nitsch, C. Mariani and W.H. Vriezen

The research presented in this thesis was initiated to gain a better understanding of the role of abscisic acid (ABA) during early fruit development. This chapter briefly summarizes what we have learned in the past years and highlights a few perspectives, which arose from the data presented here together with the data presented in literature.

ABA and fruit set

Auxin and gibberellin are important signaling molecules during fruit set, they induce fruit growth and development after pollination (Gillaspy *et al.* 1993). Several lines of evidence suggest that another hormone, abscisic acid (ABA), is an additional player in this process. Firstly, a cDNA-AFLP established that genes related to ABA were differentially expressed during fruit set. These ABA-signaling and ABA-response genes are relatively highly expressed in mature unpollinated ovaries and their expression decreases after pollination (Vriezen *et al.* 2008). Secondly, it was shown in chapter two that the content of ABA in unpollinated ovaries is high and decreases significantly in the first days after pollination. Moreover, the content of ABA is actively adjusted. Gene-expression data together with ABA-metabolite levels indicate that after pollination ABA is instantly catabolized into PA and DPA via 8`-hydroxylation. Most likely this is induced by the increase in auxin levels, as we showed that the mRNA levels of *SlCYP707A1* (encoding for the most important ABA-8`hydroxylase) increase strongly after auxin application as it does after pollination (chapter 2). A mechanism for rapid removal of ABA after pollination is thus present in the ovary, suggesting that ABA hinders fruit set, or at least is no longer required during fruit growth.

In contrast to the observations described above, a function for ABA during fruit set could not be conclusively demonstrated yet. In chapter three the isolation and characterization of a *notabilis-flacca* double mutant was described, which has very low ABA levels due to two mutations in enzymes involved in the ABA biosynthetic route. However, because the *notabilis* and *flacca* single mutants have different genetic backgrounds that showed large difference in fruit set, it proved to be extremely difficult to draw conclusions on the effect of reduced ABA levels on fruit set under the conditions we tested. A second approach (chapter four) involved the production of ABA-hypersensitive plants by silencing a *RPN10*-homologue in tomato (*SlRPN10*). The flowers of transgenic RNAi plants displayed delayed senescence, but no differences in fruit set percentages were detected. In addition to this, *SlRPN10* is not differentially expressed before and after pollination. Therefore, these observations seem to suggest that the proteasome/RPN10-mediated-route of ABA signaling most likely is not

involved in signaling during fruit set. Since there is substantial evidence that ABA signaling occurs via different routes (Finkelstein *et al.* 2002; Nambara and Marion-Poll 2003), it is not to be expected that all ABA signaling components are involved in every process that involves ABA. Therefore, it is not excluded that ABA may influence fruit set via another signaling route. This signaling route might include the action of *SlPP2C1*, which is differentially expressed during fruit set. The gene seems to be a negative regulator of the ABA responses in tomato, because transgenic tomato plants that have reduced levels of *SlPP2C1* were shown to be hypersensitive to ABA (chapter five). Possibly, further analysis of these transgenic plants is a very promising way to delineate the role of ABA in fruit set.

ABA and dormancy

The development of the fruits truly starts with the development of the ovary. The cells in the ovary divide and differentiate during this process and the ovary increases in size. Strikingly, at maturity the ovary does not directly develop further into a fruit but awaits fertilization. If no fertilization occurs, the ovary will abscize. Thus, there seems to be a growth arrest in the mature unpollinated ovary. This is confirmed by the expression data of cell cycle genes (Vriezen *et al.* 2008). Additionally, the dormancy marker *SlDRM1* is expressed specifically at ovary maturity (chapter 6). Therefore, the state of the mature ovary seems to be dormant. Dormancy is a period of arrested growth and development, which is often associated with unfavorable environmental conditions and it is found (among others) in seeds, apical buds and axillary buds.

Dormancy is regulated by a complex network of different hormones interacting with each other. In seeds, tubers and apical buds, ABA is important for the induction and maintenance of dormancy (Suttle 2004; Ruttink *et al.* 2007; Finkelstein *et al.* 2008). In axillary buds auxin produced in the apex inhibits bud outgrowth (Ongaro and Leyser 2007) together with the recently identified strigolactones (Gomez-Roldan *et al.* 2008; Umehara *et al.* 2008). However, there are also several indications that ABA is an additional inhibitor in this process (Shimizu-Sato and Mori 2001; Cline and Oh 2006). In agreement with the important role of ABA in dormancy induction, ABA can also directly influence the cell cycle via suppression of *CDKA1* expression (Smalle *et al.* 2003) or stimulation of *ICK1* (an inhibitor of CDK action) expression (Wang *et al.* 1998). Hereby, ABA can inhibit cell division and thus might induce a growth arrest. In the mature tomato ovary high levels of ABA are present concomitant with “dormancy” induction (chapter 2), suggesting a similar function for ABA in

this process. The release from dormancy has been studied extensively in seeds and it is determined by the balance between ABA (a growth inhibitor) and gibberellins (growth stimulators) (Finkelstein *et al.* 2008; Finch-savage and Leub-metzer 2006). However, not only gibberellins are antagonists of ABA in seed dormancy. Auxin also stimulates seed germination via the ARF10-miR160 interaction, which is at least partly regulating ABA sensitivity (Liu *et al.* 2007b). In the ovary the growth arrest which is observed before pollination can be broken by the application of auxins or gibberellins (Gilaspy *et al.* 1993). Moreover, concomitant with a decrease in ABA levels after pollination (chapter 2), the content of auxins and gibberellins increases (Koshioka *et al.* 1994: Mapeli *et al.* 1978), indicating a change in the balance between these hormones during fruit initiation. This similarity with the hormonal regulation of dormancy release suggests that the hormone balance between ABA and auxin/gibberellin might control growth and development in tomato ovaries after pollination. If this is true, the level of ABA is less important, as compared to its balance with other hormones. Considering this, it might not be so surprising that ABA and fluridone treatments were unsuccessful to influence fruit set. Additionally, it is not completely known how effectively these exogenous treatments may influence endogenous ABA levels. Moreover, the increase in the levels of auxin and gibberellin after pollination might be so large that an excess of hormones accumulates, thus any level of ABA may not be able to balance these stimulating substances, unless it is extremely high. A system to finely manipulate the hormone balance in ovaries may in the future help to unravel the role of ABA in fruit set.

ABA and the ovule

Interestingly, the research presented here highlighted that ABA might have the most pronounced effects in the ovules. *SlCYP707A1*, the gene encoding for the ABA catabolic enzyme, was localized specifically in the ovules where it increased several hundred-fold after pollination (chapter 2). This suggests that the reduction in ABA levels after pollination is largest and perhaps most important in the ovules. It also might suggest that before pollination ABA levels are highest in the ovules. In Arabidopsis and cucumber ovaries at maturity ABA was indeed mainly localized in the inner and outer integument of the ovules (Peng *et al.* 2006). In addition, the *SlPP2C1* gene, most likely a negative regulator of the ABA response, is least expressed in the ovules before pollination (chapter 5). This might indicate that the ABA sensitivity before pollination thus is relatively high in the ovules. Above all this, the

dormancy marker gene *SlDRM1* was also mainly localized in the ovules, suggesting it would have a function there (chapter 6). Strong correlation between *DRM*-expression and growth arrest has been demonstrated (Park and Han 2003; Stafstrom *et al.* 1998). Therefore, it seems that the ovules are most "dormant". Exactly there ABA is also expected to function most strongly.

ABA and environmental factors

Besides dormancy, ABA is well-known for its function in abiotic stress responses (Seki *et al*. 2007; Wasilewska *et al.* 2008). Although we sometimes tend to consider the ABA mediated effects of stress as unfavorable, ABA is part of a regulatory network which enables plants to survive stress conditions. It is known that ABA can induce the expression of genes involved in stress adaptations such as the production of osmo-protectants (sugars, dehydrins, and proteins). Interestingly, the induction of similar genes also occurs during developmental processes, for instance during the induction of seed or apical bud dormancy where it renders tissues desiccation or cold tolerant (Finkelstein *et al.* 2008; Ruttink *et al.* 2007). Strikingly, in the mature unpollinated ovary also several genes related to stress-adaptation were highly expressed such as *DREBs*, dehydrins and a salt-tolerance gene, but also ethylene biosynthesis and ethylene responses related genes (Vriezen *et al.* 2008). Similarly, in rice it was also found that many genes related to drought stress adaptations were highly expressed in unpollinated pistils and lowly expressed after pollination/fertilization (Lan *et al.* 2005). It thus seems that the mature unpollinated ovary is adapted to stress conditions. This was also implied by the high ABA levels in unpollinated ovaries which were comparable to the levels in stressed leaves (chapter 3). Besides the proposed function for ABA in the induction and maintenance of dormancy in mature unpollinated ovaries, ABA might additionally induce mechanisms to protect the mature ovary before pollination. The plant may invest in such protective mechanisms to increase its chances for reproduction. In addition, if the mature unpollinated ovary is dormant, it might also be metabolically inactive and this might hamper a quick response of the ovary to environmental factors if necessary. Such a stress-adaptation mechanism may form another similarity with seed and bud dormancy, where ABA also induces stress-adaptations.

Although not conclusive, I have approached in my thesis a novel view of the mature tomato ovary before pollination, in which it arrests its growth and becomes dormant. ABA may very well induce this dormant state and protect the ovary before pollination.

Summary / Samenvatting

Summary

A major and nutritionally important part of the human diet is composed of fruits or fruit-derived products. Additionally, fruits are important in the life cycle of plants, since they protect the seeds during their development and allow seed dispersal. Therefore, there has been considerable research on fruit development, for which tomato has been used as a model system for physiological and molecular studies for a long time. The process of fruit development truly starts with ovary development. Many cell divisions are made in the ovary before it reaches its mature state at flower anthesis. Strikingly, when the ovary is mature there is a temporary reduction in cell division activity, and the ovary will not grow further into a fruit unless it is fertilized. Additionally, dormancy markers are highly expressed in the mature ovary. Therefore, this temporary arrest in growth and development in the ovary seems to resemble dormancy as it is found in axillary buds and seeds. The first phase following ovary development is called fruit set or fruit initiation and is defined as the stage at which the ovary is committed to abort or to proceed with further cell division and fruit development. It has long been known that application of hormones such as auxins or gibberellins can induce fruit set in the absence of fertilization. The resulting parthenocarpic fruit growth is a clear demonstration of the importance of these hormones in fruit initiation and development. Research undertaken to discover some of the molecular players during fruit set resulted in the discovery of many genes related to auxin and gibberellin signaling. However, surprisingly, also many genes related to stress and abscisic acid (ABA) were found to be differentially expressed. The research presented in this thesis was aimed to gain more insight in the role of abscisic acid during fruit set.

ABA response- and signaling-genes were highly expressed in the mature ovary before pollination and reduced significantly after pollination. In **chapter two** we showed that ABA hormone levels also decrease after pollination. mRNA levels of *LeNCED1*, encoding for an enzyme that catalyzes the first committed step of ABA biosynthesis, decrease after pollination, suggesting that ABA biosynthesis is reduced. Additionally, the mRNA levels of *SlCYP707A1*, which we indirectly proved to encode an ABA-8' hydroxylase, increased strongly. This suggests that ABA levels are reduced due to increased ABA-8` hydroxylation, which results in the formation of phaseic acid (PA) and dihydrophaseic acid (DPA). Indeed the DPA levels were three-fold higher in pollinated ovaries, strengthening our hypothesis. Over-expression of *SlCYP707A1* resulted in plants that had lower ABA levels and that under water stress displayed severe wilting.

We tried to obtain more information about the role of ABA in fruit development by using three different strategies. **Chapter three** describes the isolation of a *notabilis-flacca* (*not/flc*) double mutant and the characterization of the ABA-deficient single and double mutants. Unfortunately, because the *notabilis* and *flacca* single mutants have different genetic backgrounds that showed large difference in fruit set, it proved to be extremely difficult to draw conclusions on the effect of reduced ABA levels on fruit set under the conditions we tested. We did find that the response measured for plant growth, leaf-surface area, wilting and ABA related gene expression in the different ABA-deficient lines was in accordance with ABA levels. Moreover, fruit size was smaller in the ABA-deficient lines, most likely due to the reduced cell size that we observed in *not/flc* double mutants. In **chapter four,** another strategy was described that involved the production of ABA-hypersensitive plants by silencing a *AtRPN10*-homologue in tomato (*SlRPN10*). The flowers of transgenic *SlRPN10*-RNAi plants displayed delayed senescence in the absence of pollination and slower progression of senescence after pollination. We also measured that ethylene emission rates were lower in the transgenic flowers before, at, and after anthesis, indicating a possible cause of the reduced senescence. Fruit set however, was not affected in these RNAi lines. In addition to the *SlRPN10*-RNAi lines we also set out to characterize transgenic tomato plants in which *SlPP2C1* is silenced or over expressed (**chapter five**). *SlPP2C1* is homologous to *AtABI1* and *AtABI2*, which code for negative regulators of the ABA response. Transgenic plants co-suppressing *SlPP2C1* display ABA-hypersensitive phenotypes in root growth, seed germination and drought responses, indicating that *SlPP2C1* codes for a negative regulator of the ABA response in tomato as well. Moreover, these plants may provide the necessary tools to study the function of ABA in mature ovaries and during fruit set.

Finally, we also studied the expression and regulation of *SlDRM1*, which is a dormancy marker, during ovary and fruit development (**chapter six**). *SlDRM1* is expressed at low levels during ovary development, but mRNA levels strongly increase at ovary maturity. Pollination, auxin and gibberellin treatments reduce the mRNA levels of *SlDRM1* and this is mediated by the 3´-UTR, suggesting that transcript stability is altered by these treatments. The mRNA is mainly localized in the ovules, more specifically in the cells surrounding the embryo sack, suggesting that here *SlDRM1* may have a function.

In **chapter seven** it is concluded that a mechanism for rapid removal of ABA after pollination is present in the ovary, suggesting that ABA hinders fruit set or at least is no longer required. The many parallels between dormancy in, for instance, seeds and axillary buds, and the status of the mature ovary before pollination, suggest that the ovary is in a

dormancy-like state as well. A possible function for ABA could therefore be the induction and maintenance of dormancy in the mature ovary as it does in other dormant tissues. The balance between stimulating substances, such as auxin and gibberellins, and ABA may then influence the out-come of fruit set.

Samenvatting

Een groot en belangrijk gedeelte van de voeding van de mens bestaat uit vruchten en daarvan afgeleidde producten. Daarnaast zijn vruchten ook belangrijk voor de levenscyclus van planten omdat zij de zaden beschermen tijdens hun ontwikkeling en zorgen voor zaadverspreiding. Er is veel onderzoek gedaan naar vruchtontwikkeling; en hiervoor is tomaat vaak gebruikt als model systeem voor fysiologische en moleculaire studies. De vruchtontwikkeling begint met de ontwikkeling van het ovarium. Een groot aantal celdelingen vindt plaats in het ovarium voordat het haar volwassen staat bereikt. Het is opvallend dat de celdelingactiviteit tijdelijk vermindert wanneer het ovarium zijn volwassen staat heeft bereikt. Het ovarium zal niet verder uitgroeien tot een vrucht tenzij er bevruchting plaatsvindt. Daarnaast blijken markergenen voor dormantie (rustperiode) hoog tot expressie te komen in het volwassen ovarium. De staat van het volwassen ovarium lijkt hierdoor vergelijkbaar met dormantie zoals we dat vinden bij zijknoppen en zaden. De fase die volgt op de ontwikkeling van het ovarium wordt vruchtzetting of vruchtinitiatie genoemd. Deze fase is gedefinieerd als het stadium waarin het ovarium de beslissing neemt om af te sterven of door te gaan met verdere celdelingen en vruchtontwikkeling. Het is al geruime tijd bekend dat het toedienen van hormonen zoals auxine of gibberelline bij afwezigheid van bevruchting toch vruchtzetting kan induceren. Soortgelijke hormoontoediening resulteren in zaadloze (parthenocarpe) vruchten en geven duidelijk aan dat deze hormonen belangrijk zijn voor vruchtinitiatie en vruchtontwikkeling. Onderzoek gericht op de ontdekking van genen die betrokken zijn bij de vruchtzetting heeft dan ook geleid tot de ontdekking van veel genen die gerelateerd bleken aan auxine of gibberelline signaalroutes. Verrassend was daarnaast de ontdekking van genen gerelateerd aan stress en abscisinezuur (ABA). Het onderzoek dat in dit proefschrift gepresenteerd wordt heeft als doel meer inzicht te verschaffen in de rol van abscisinezuur tijdens vruchtzetting.

Genen betrokken bij de ABA respons en signaalroute kwamen hoog tot expressie in het volwassen ovarium voordat bestuiving had plaatsgevonden en daarna significant minder. In **hoofdstuk twee** tonen wij aan dat ook de hormoonniveaus van ABA verminderen na bestuiving. De mRNA niveaus van *LeNCED1*, het gen dat codeert voor een enzym dat de eerste stap van de biosynthese van ABA katalyseert, waren lager na bestuiving, dit suggereert dat de biosynthese van ABA na bestuiving vermindert. Na bestuiving werd een veel hoger mRNA niveau gemeten van *SlCYP707A1,* het gen waarvan wij indirect hebben aangetoond dat het codeert voor een ABA-8'hydroxylase; een enzym dat de afbraak van ABA katalyseert.

Dit suggereert dat de hormoonniveaus van ABA na bestuiving verminderen door ABA-8' hydroxylatie, waarbij de afbraakproducten phaseic acid (PA) en dihydrophaseic acid (DPA) worden gevormd. DPA niveaus bleken inderdaad verdriedubbeld na bestuiving, wat onze hypothese bevestigt. Over-expressie van het gen *SlCYP707A1* in tomaat resulteerde in planten die lagere hormoon niveaus van ABA bevatten en erg gevoelig waren voor droogtestress.

Op drie verschillende manieren hebben we vervolgens geprobeerd meer informatie te verkrijgen over de rol van ABA tijdens vruchtontwikkeling. In **hoofdstuk drie** wordt de isolatie van *notabilis-flacca* dubbelmutanten en de karakterisering van deze ABA-deficiente dubbel- en enkelmutanten besproken. Dit zijn mutanten die minder ABA biosynthese hebben en dus lagere ABA niveaus. Helaas bleken er grote verschillen in vruchtzetting te bestaan tussen de twee genetische achtergronden van de enkelmutanten *notabilis* en *flacca*. Hierdoor was het erg moeilijk om conclusies te trekken over het effect dat verminderde ABA niveaus op vruchtzetting heeft. We vonden wel een correlatie tussen de hormoonniveaus van ABA en plant groei, blad oppervlakte, verdroging en de expressie van genen gerelateerd aan ABA, in de verschillende ABA deficiente lijnen. Daarnaast bleek de vruchtgrootte kleiner in de ABA deficiente lijnen, waarschijnlijk veroorzaakt door de kleinere cellen in de vruchten van de *notabilis-flacca* dubbel mutant. In **hoofdstuk vier** wordt een andere strategie beschreven waarbij ABA hypergevoelige planten werden gecreëerd, door de expressie van een gen dat homoloog is aan *AtRPN10 (SlRPN10*) te onderdrukken met behulp van RNAi. De bloemverwelking in transgene *SlRPN10*-RNAi planten bij afwezigheid van bestuiving was uitgesteld. Na bestuiving was het verwelkingproces vertraagd. We hebben gemeten dat de snelheid van ethyleen emissie lager was in transgene bloemen voor, tijdens en na bloemopening. Dit kan een mogelijke verklaring zijn voor de verminderde verwelking in deze bloemen. Vruchtzetting was echter niet veranderd in de RNAi-lijnen. Behalve *SlRPN10*-RNAi lijnen hebben we ook de expressie van het *SlPP2C1* gen verhoogd en onderdrukt in transgene tomaten planten (**hoofdstuk vijf**). *SlPP2C1* is homoloog aan *AtABI2* en *AtABI2*, genen die coderen voor negatieve regulatoren van de respons op ABA. In transgene planten waarin de expressie van het *SlPP2C1* gen onderdrukt werd, waren ABA overgevoelige fenotypes zichtbaar tijdens de wortel groei, zaadkieming en droogte respons. Deze resultaten geven aan dat *SlPP2C1* ook codeert voor een negatieve regulator van de respons op ABA. De trasngene planten kunnen in de toekomst de mogelijkheid bieden om de functie van ABA in volwassen ovaria en tijdens vruchtzetting te bestuderen.

Als laatste hebben we ook de expressie en regulatie van *SlDRM1*, een marker gen voor dormantie, bestudeerd tijdens ovarium, - en vruchtontwikkeling (**hoofdstuk zes**). Lage

mRNA niveaus van *SlDRM1* zijn aanwezig in het ovarium tijdens haar ontwikkeling, de mRNA niveaus stijgen echter sterk wanneer het ovarium volwassen wordt. Bestuiving, auxine en gibberelline behandeling verminderden de mRNA niveaus van *SlDRM1*. Dit bleek gemedieerd te zijn door het 3'UTR (een deel van het mRNA dat niet vertaald wordt in eiwit). Het 3'UTR is belangrijk voor de stabiliteit van het mRNA en de resultaten suggereren dan ook dat de stabiliteit van het mRNA verlaagd wordt door deze behandelingen. Het mRNA is vooral gelokaliseerd in de ovules, specifiek in de cellen rondom de embryozak dit suggereert dat *SlDRM1* daar zijn functie uitoefent.

In **hoofdstuk zeven** wordt geconcludeerd dat een mechanisme voor snelle verwijdering van ABA na bestuiving aanwezig is in het volwassen ovarium. Dit suggereert dat ABA de vruchtzetting verhindert of dat ABA in ieder geval niet langer noodzakelijk is. De velen parallellen tussen dormantie in, bijvoorbeeld zijknoppen en zaden, en de staat van het volwassen ovarium voor bestuiving suggereren dat het ovarium eveneens in een dormante staat verkeerd. Daarom zou een mogelijke functie voor ABA de inductie en het behoud van dormantie in het volwassen ovarium kunnen zijn, zoals het ook deze functie heeft in andere dormante weefsels. De balans tussen stimulerende (auxine en gibberelline) en inhiberende substanties (ABA) kan wellicht de uitkomst van de vruchtzettingfase beïnvloeden.

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Dankwoord en curriculum vitae

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zei dat je ging vertrekken, help onze lab-encyclopedie ging er van door. Als labmanager regelde je alles en je moleculaire kennis was onovertroffen. Dat heeft me de eerste twee jaar geweldig goed geholpen. Het was dan ook echt even lastig toen je weg was, gelukkig was Jan er nog. Bedankt voor alle hulp en adviezen. Peter, van fysioloog naar moleculair bioloog en lab manager is niet altijd even makkelijk, maar je doet het lang niet slecht. Bedankt voor je inzet. Thikra je ging van student, naar analist, naar collega-aio. Bedankt voor al je hulp en gezelligheid en heel veel succes de komende jaren. Ook Gerco en de mensen van Plant Genetica; Anneke, Veena, Partha, Janny, Jan, Michiel, en Tom wil ik bedanken voor de adviezen tijdens de woensdagochtend bespreking en natuurlijk voor de gezelligheid op het lab, de borrels en feestjes.

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Curriculum vitae

Lisette Maria Catharina Nitsch werd op 25 november 1981 geboren in Brunssum (Zuid-Limburg). Ze deed haar VWO opleiding aan het Rombouts college in Brunssum en studeerde in 1999 cum laude af. In dat zelfde jaar begon zij aan haar studie biologie aan de toen nog Katholieke Universiteit Nijmegen. Haar eerste masterstage deed zij bij de afdeling organismale dierfysiologie onder leiding van prof. dr. Gert Flik en dr. Peter Klaren. Zij deed onderzoek naar de invloed van het schildklierhormoon op de osmotische regulatie van vissen. Daarna ging zij naar het lab van prof. dr. Juan-Miguel Mancera aan de Universidad de Cadiz (Zuid-Spanje) waarzij de ontwikkeling van de schildklier in zeebrassem bestudeerde. Haar tweede afstudeerstage werd uitgevoerd op de afdeling celbiologie van de plant onderleiding van prof. dr. Celestina Mariani en drs. Flora Ismael. Hier verrichte zij onderzoek aan de vivipare zaden van de mangrove *Rhizophora Mucronata* (Lam). In september 2004 begon Lisette aan haar promotieonderzoek naar de rol van het hormoon abscisinezuur in de vruchtzetting van tomaat, waarvan de resultaten in dit boekje beschreven staan. Tijdens haar promotietrajekt presenteerde zij haar onderzoek op verschillende internationale congressen waaronder de FESB-meeting in Lyon (Frankrijk, 2006), de IPGSA-meeting in Puerto Vallarta (Mexico, 2007) en de Solanacea-meeting in Keulen (Duitsland, 2008). Een onderdeel van het onderzoek werd uitgevoerd bij het Rothamstead Research Centre in het lab van dr. Peter Hedden in Harpenden (United Kingdom).

Tijdens haar biologie-studie werd zij lid van de seminarcommissie van de biologie-studenten vereniging BeeVee. Zij was vier jaar lid van deze commissie waarvan een jaar als voorzitter. In 2006 zette zij samen met collega junior-onderzoekers de seminarcommissie van het onderzoeksinstituut, the institute for water and wetland research (IWWR), op. Zij organiseerde maandelijks seminars, jaarlijks een AIO-dag, en enkele andere activiteiten zoals een loopbaan orientatie voor junior-onderzoekers. Daarnaast begeleidde ze een aantal bachelor en masterstudenten tijdens hun stage bij de afdeling celbiologie van de plant. Ook gaf zij een aantal gastcolleges en hielp ze mee met de practica bij enkele cursussen gegeven door deze afdeling. In 2007 volgde ze de cursus didactiek in de praktijk van het IOWO aan de Radboud University Nijmegen.

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